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(54) Title: INHIBITORS OF CELL PROLIFERATION, THEIR PREPARATION AND USE

(57) Abstract

Disclosed is the characterization and purification of DNA encoding numerous polypeptides factors useful for the inhibition of cell (particularly, Schwann cell) proliferation. These factors are useful for the treatment of neural tumors. Also disclosed are the DNA sequences encoding novel polypeptides which may have use as agents which inhibit cell proliferation. Methods for the synthesis, purification, and testing of both known and novel polypeptides for their use as therapeutic and diagnostic aids in the treatment of diseases are also provided. Methods are also provided for the use of these polypeptides for the preparation of antibody probes. Such probes have diagnostic and therapeutic use in diseases involving neural and glial cells.

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INHIBITORS OF CELL PROLIFERATION, THEIR PREPARATION AND USE
Background of the Invention

The invention relates to compounds which are
5   inhibitors of cell proliferation, having antiproliferative
activity on a variety of cell types.

Many vertebrate cell types respond to diffusible
growth factors as stimuli which regulate proliferation.
A number of these growth factors and their cognate
10 receptors have been purified, and the genes encoding them
have been cloned and characterized (Sporn and Roberts eds.
(1991) Peptide Growth Factors and their Receptors I and
II. Springer-Verlaz, New York). Many cancers, which are
diseases of cell proliferation, involve genetic
15 modifications which affect the nature of the growth
factor-receptor interaction. Such modifications can result
in unregulated stimulation of proliferation in the receptor
bearing target cell. Additionally, certain tumors of the
nervous system involve the regulation of proliferation of
20 cells from both the central and peripheral nervous systems.

The glial cells of vertebrates constitute the
specialized connective tissue of the central and peripheral
nervous systems. Important glial cells include the
peripheral Schwann cells which provide both the metabolic
25 support for neurons and the myelin sheathing around the
axons of certain peripheral neurons, thereby forming
individual nerve fibers. Schwann cells support neurons and
provide a sheath effect by forming concentric layers of
membrane around adjacent neuron axons, twisting as they
30 develop around the axons. These myelin sheaths are a
susceptible element of many nerve fibers. Damage to
Schwann cells, or failure in growth and development, can be
associated with significant demyelination or nerve
degeneration characteristic of a number of peripheral
35 nervous system diseases and disorders. In the development

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of the nervous system, it has become apparent that cells require various factors to regulate their division and growth. Several regulators of Schwann cell proliferation and differentiation have been identified. Such factors

5 play an important role in both the development and the regeneration (following injury) of the peripheral nervous system.

Brockes et al. ((1984) J. Neuroscience 4:75-83) describe a protein growth factor present in extracts from

10 bovine brain and pituitary tissue, termed Glial Growth Factor (GGF). This factor stimulates cultured rat Schwann cells to divide against a background medium containing ten percent fetal calf serum. GGF has been described as having a molecular weight of 31 KD and readily forming dimers.

15 Brockes ((1987) Meth. Enz. 147:217-225) describes a Schwann cell-based assay for 31 kD GGF and purification using reversed phase HPLC.

The J. Neuroscience article of Brockes et al., supra, describes methods of purification of GGF to apparent

20 homogeneity. In brief, one large-scale purification method described involves extraction of the lyophilized bovine anterior lobes and chromatography of material obtained thereby, using NaCl gradient elution from CM cellulose. Gel filtration is then carried out with an Ultrogel column,

25 followed by elution from a phosphocellulose column, and finally, small-scale SDS gel electrophoresis. Alternatively, the CM-cellulose material was applied directly to a phosphocellulose column, fractions from the column were pooled and purified by preparative native gel

30 electrophoresis, followed by a final SDS gel electrophoresis.

Brockes et al. ((1980) J. Biol. Chem. 255:8374-8377) observe that in gel filtration experiments the major peak of growth factor activity is observed to migrate with a

35 molecular weight of 56 KD, whereas in the first of the

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above-described procedures activity was predominantly observed at molecular weight 31 KD. They report that the GGF dimer is largely removed as a result of the gradient elution from CM-cellulose in this procedure.

5   Benveniste et al. ((1985) PNAS 82:3930-3934) describe a T lymphocyte-derived glial growth promoting factor. This factor, under reducing conditions, exhibits a change in apparent molecular weight on SDS gels.

         Kimura et al. ((1990) Nature 348:257-260) describe
10 a factor they term Schwannoma-derived growth factor (SDGF) obtained from a sciatic nerve sheath tumor. The authors state that SDGF does not stimulate the incorporation of tritium-labelled TdR into cultured Schwann cells under conditions where, in contrast, partially purified pituitary
15 fraction containing GGF is active. SDGF has an apparent molecular weight between 31 KD and 35 KD.

         Davis et al. ((1990) J. Cell. Biol. 110:1353-1360) describe the screening of a number of candidate mitogens. The chosen candidate substances being examined for their
20 ability to stimulate DNA synthesis in Rat Schwann cells in the presence of 10% FCS (fetal calf serum), with and without forskolin. One of the factors tested, GGF-carboxymethyl cellulose fraction (GGF-CM), was mitogenic in the presence of FCS, with and without
25 forskolin. It was also observed that in the presence of forskolin platelet derived growth factor (PDGF) is a potent mitogen for Schwann cells. Previous to this finding, PDGF was not thought to have a mitogenic effect on Schwann cells.

30   Holmes et al. ((1992) Science 256:1205) and Wen et al. ((1992) Cell 69:559) demonstrate that DNA sequences which encode proteins which bind to a receptor (p185^{erbB2}) are associated with several human tumors.

         The p185^{erbB2} protein is a 185 kilodalton membrane
35 spanning protein with tyrosine kinase activity. The

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protein is encoded by the *erbB2* proto-oncogene (Yarden and
Ullrich. (1988) Ann. Rev. Biochem. $\underline{57}$:443). The *erbB2*
gene, also referred to as HER-2 (in human cells) and *neu*
(in rat cells), is closely related to the receptor for
5 epidermal growth factor (EGF). Recent evidence indicates
that proteins which interact with (and activate the kinase
of) p185^{erbB2} induce proliferation in the cells bearing
p185^{erbB2} (Holmes et al. (1992) Science $\underline{256}$:1205; Dobashi et
al. (1991) Proc. Natl. Acad. Sci. $\underline{88}$:8582; and Lupu et al.
10 (1992) Proc. Natl. Acad. Sci. $\underline{89}$:2287).

      Although ligands have been identified which
stimulate proliferation of cells with certain receptors
(e.g., the p185^{erbB2} receptor), there exists a need to
identify and isolate factors which act as inhibitors of
15 cell proliferation at these receptor sites. Such
inhibitors could be used for the purpose of treating cell
proliferative disorders (e.g., neoplasms).

Summary of the Invention

      In general, the invention provides methods for
20 inhibiting proliferation of cells, including cells of the
nervous system. The antiproliferative factors of the
invention are alternative splicing products and fragments
thereof of the DNA encoding the GGF/p185^{erbB2} family of
proteins.

25   The invention also provides a DNA sequence encoding
a glial growth inhibitory factor; the sequence is included
in the clone pGGF2HBS11 (ATCC Deposit No. 75347).

      The peptide encoded by this clone is also a part of
the invention. The invention further includes a peptide
30 comprising a peptide encoded by the E sequence (SEQ ID Nos.
137 and 163) and at least a portion of the peptides encoded
by brain derived DNA sequences flanking the E encoding
sequences on clone pGGF2HBS11 (ATCC Deposit No. 75347).
Preferably, the E encoded polypeptide sequence lacks 48

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amino acids on the amino-terminal end and includes between
20 and 100 or, more preferably, between 25 and 70 amino
acids of flanking the E encoded polypeptide. In addition,
the E encoded polypeptide may be flanked by between 30 and
5 50, or, more preferably, between 35 and 45 amino acids on
the carboxy terminal side of the E encoded segment. The
sequences flanking the E encoded polypeptide are encoded by
the DNA sequences flanking the E sequence present in clone
pGGF2HBS11 (ATCC Deposit No. 75347).

10   Specifically, the invention also provides a method
for inhibiting cell proliferation *in vitro* or *in vivo*
comprising contacting the cell with
        a) a polypeptide defined by the formula

                        VYBAZWX
15   wherein VYBAZWX is composed of the polypeptide
segments shown in Figure 31 (SEQ ID Nos. 136-139, 141-147,
160, 161); wherein V comprises F, or is absent; wherein Y
comprises polypeptide segment E, or is absent; wherein Z
comprises polypeptide segment G or is absent; wherein W
20 comprises C or is absent; and wherein X comprises
polypeptide segments C/D HKL, C/D H, C/D HL, C/D D, C/D'
HL, C/D' HKL, C/D' H, C/D' D, C/D C/D' HKL, C/D C/D' H, C/D
C/D' HL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D'
D' H, C/D' D' HL, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D'
25 HL, H, HK, HKL, or C/D C/D' D' HKL;
        b)   a polypeptide comprising FBA polypeptide
segments having the amino acid sequences shown in Figure 31
(SEQ ID Nos. 136, 138, 139);
        c)   a polypeptide comprising FBA' polypeptide
30 segments having the amino acid sequences shown in Figure 31
(SEQ ID Nos. 136, 138, 140, 168);
        d)   a polypeptide comprising FEBA polypeptide
segments having the amino acid sequences shown in Figure 31
(SEQ ID Nos. 136-139); or

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e)   a   polypeptide   comprising   FEBA'   polypeptide
segments having the amino acid sequences corresponding to
polypeptide segments shown in Figure 31 (SEQ ID Nos. 136-
138, 140, 168);

5   f)   a   polypeptide   comprising   EBA'   polypeptide
segments having the amino acid sequences corresponding to
polypeptide segments shown in Figure 31 (SEQ ID Nos. 136,
138, 140, 168); or

g)   a   polypeptide   comprising   a   portion   of   the   E
10 sequence (SEQ ID Nos. 137 and 163) and flanked by new
sequence not contained in F, B, A, C/D, C/D', D, D', HK or
L and contained in clone pGGF2HBS11, ATCC Deposit No.
75347; or to glial cells (i.e., astrocytes and microglial
cells of the central and peripheral nervous system and
15 Schwann cells of the peripheral nervous system).

The invention also provides a method of inhibiting
proliferation of cells, including cells of the nervous
system, by a method which consists of contacting the cells
with a compound which specifically binds the p185^{erbB2}
20 receptor of the cell type.

Also   included   is   the   method   comprising   the
administration of any of the above mentioned peptides when
the   peptides   are   administered   in   the   treatment   or
prophylaxis of a nervous disease or disorder.   Further
25 included in the invention is the method of administering
any   of   the   above   mentioned   peptides   when   the   cell   is
present in a mammal and the contacting of the cell is
carried out by the administration of the peptide to the
mammal   for   the   prophylaxis   or   treatment   of   a
30 pathophysiological condition in the mammal which involves
the stated cell.   Also included is the use of the method,
as stated above, wherein the condition involves a disease
of   cell   proliferation,   such   as   a   tumor,   and   more
specifically, where the condition involves peripheral nerve

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damage caused by a tumor of the nervous system. Also a
part of the invention is the administration of the
inhibitory factors for the purpose of increasing
myelination of existing or regenerated neural tissue.

5   Further included as a part of the invention are
methods comprising administration of any of the above
mentioned polypeptides to a cell when the cell is present
in a mammal and the contacting of the cell is carried out
by administering the peptide to the mammal for the
10 prophylaxis or treatment of a condition which involves one
of the following conditions: a tumor of the Schwann cells,
for example, neurofibromatosis, malignant Schwannomas or
neurofibrosarcomas; a meningioma; a bilateral acoustic
neuroma; an astrocytoma; a retinoblastoma; a neuroglioma;
15 a neuroblastoma; an adenocarcinoma; or a glioma, by the
method comprising administering to the mammal an effective
amount of a polypeptide, as defined above.

          The invention also includes a method for producing
an antibody specific for a polypeptide, consisting of
20 immunizing a mammal with a polypeptide selected from the
above listed polypeptides, or a fragment thereof, and
purifying the antibody from the tissue of the animal, or
from a hybridoma made using the tissue.

          Furthermore, the invention provides a method for
25 detecting, in a sample, the presence of a molecule capable
of binding to a receptor which binds to a polypeptide
selected from the above mentioned polypeptides, and
contacting the sample with the polypeptide together with
the receptor, and detecting inhibition of the binding of
30 the polypeptide to the receptor as an indication of the
presence of a receptor binding molecule in the sample. The
invention also provides methods for determining whether
such a competitive inhibitor is an antagonist or agonist of
receptor function.

35   Thus, factors useful in the methods of the invention

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are:-

(a) basic polypeptide factors having antiproliferative activity when contacted with cells, including cells of the nervous system and specifically
5 Schwann cells, and containing within their amino acid sequences one or more of the following peptide sequences:

F K G D A H T E
A S L A D E Y E Y M X K
T E T S S S G L X L K
10 A S L A D E Y E Y M R K
A G Y F A E X A R
T T E M A S E Q G A
A K E A L A A L K
F V L Q A K K
15 E T Q P D P G Q I L K K V P M V I G A Y T
E Y K C L K F K W F K K A T V M
E X K F Y V P
K L E F L X A K; and

(b) basic polypeptide factors capable of inhibiting
20 the division of cells, including cells of the nervous system and particularly Schwann cells, and containing within their amino acid sequences, respectively, one or more of the following peptide sequences:

V H Q V W A A K
25 Y I F F M E P E A X S S G
L G A W G P A F P V X Y
W F V V I E G K
A S P V S V G S V Q E L Q R
V C L L T V A A L P P T
30 K V H Q V W A A K
K A S L A D S G E Y M X K
D L L L X V

The peptide sequences set out above, derived from lower and higher molecular weight polypeptide factors
35 described in detail below, are also aspects of this

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invention in their own right. These sequences are
potentially useful as therapeutics, probes for large
polypeptide factors, for investigating, isolating or
preparing such factors (or corresponding gene sequences)
5 from a range of different species, or preparing such
factors by recombinant technology, and in the generation of
antibodies (preferably monoclonal), by conventional
technologies, which are themselves useful as investigative
tools and potential medicaments. Such antibodies are
10 included within this invention. The invention also
includes inhibitors of cell proliferation encoded by gene
sequences obtainable using the peptide sequences of the
invention.

The invention further includes methods for use of a
15 polypeptide factor having cell, including cells of the
nervous system, antiproliferative activity and including an
amino acid sequence encoded by:

(a)   a DNA sequence shown in any one of Figures
       28a, 28b or 28c (SEQ ID Nos. 133-135
20   respectively);
(b)   a DNA sequence shown in Figure 22 (SEQ ID No.
       89);
(c)   the DNA sequence represented by nucleotides
       281-557 of the sequence shown in Figure 28a
25   (SEQ ID No. 133); or
(d)   a DNA sequence hybridizable to any one of the
       DNA sequences according to (a), (b) or (c).

While the present invention is not limited to a
particular set of hybridization conditions, the following
30 protocol gives general guidance which may, if desired, be
followed:

Thus, DNA probes may be labelled to high specific
activity (approximately 10^8 to 10^9 dpm ^{32}P per μg) by
nick-translation or by PCR reactions according to
35 Schowalter and Sommer ((1989) Anal. Biochem. 177:90-94) and

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purified by desalting on G-150 Sephadex columns. Probes
may be denatured (10 minutes in boiling water followed by
immersion into ice water), then added to hybridization
solutions of 80% buffer B (2g polyvinylpyrolidine, 2g
5 Ficoll-400, 2g bovine serum albumin, 50μl 1M Tris HCL (pH
7.5), 58g NaCl, 1g sodium pyrophosphate, 10g sodium dodecyl
sulfate, 950μl H_2O) containing 10% dextran sulfate at 10^6
dpm ^{32}P per μl and incubated overnight (say, 16 hours) at
60°C. The filters may then be washed at 60°C, first in
10 buffer B for 15 minutes followed by three 20-minute washes
in 2X SSC, 0.1% SDS then one for 20 minutes in 1x SSC, 0.1%
SDS.

The methods of the invention take advantage of the
fact that a Glial Growth Factor and the p185^{erbB2} ligand
15 protein are encoded by the same gene. A variety of
messenger RNA splicing variants (and their resultant
proteins) are derived from this gene, and many of these
products exhibit p185^{erbB2} binding. This binding may result
in either cell proliferation or cessation of cell division.
20 At least two of the gene products (GGFI and GGFII) have
been used to induce Schwann cell mitogenic activity. This
invention employs some of the known products of the
GGF/p185^{erbB2} ligand gene (described in the references
listed above) as inhibitors of cell proliferation and, more
25 specifically, as inhibitors of glial cell proliferation.

This invention also relates to other, not yet
naturally isolated splicing variants of the Glial Growth
Factor gene. Figure 30 shows the known patterns of splicing
derived from polymerase chain reaction experiments (on
30 reverse transcribed RNA) and analysis of cDNA clones (as
presented within) and derived from what has been published
as sequences encoding p185^{erbB2} ligands (Peles et al. (1992)
Cell 69:205 and Wen et al. (1992) Cell 69:559). These
patterns, as well as additional patterns disclosed herein,
35 represent probable existing splicing variants.

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Thus other aspects of the invention are :

Methods for the use of a series of human and bovine polypeptide factors having cell antiproliferative activity, including the inhibition of the division of cells of the
5 nervous system, such as Schwann cells. Such peptide sequences are shown in Figure 31-34, (SEQ ID Nos. 136-137), respectively.

The human peptide sequences described above and presented in Figures 31-34, SEQ ID Nos. 136-137
10 respectively, represent a series of splicing variants which can be isolated as full length complementary DNA's (cDNA's) from natural sources (cDNA libraries prepared from the appropriate tissues) or assembled as DNA constructs with individual exons (e.g., derived as separate exons) by one
15 skilled in the art.

Other compounds, in particular, peptides, which bind specifically to the $p185^{erbB2}$ receptor can also be used according to the invention as inhibitors of glial cell proliferation. A candidate compound can be routinely
20 screened for $p185^{erbB2}$ binding, and, if it binds, can be screened for inhibition of cell proliferation using the methods described herein.

The invention includes the use of any modifications or equivalents of the above polypeptide factors which do
25 not exhibit a significant reduction in the stated inhibitory activity. For example, modifications in which amino acid content or sequence is altered without substantially adversely affecting inhibitory activity are included. By way of illustration, in EP-A 109748 muteins
30 of native proteins are disclosed in which the possibility of unwanted disulfide binding is avoided by replacing any cysteine in the native sequence which is not necessary for biological activity with a neutral amino acid. The statements of effect and use contained herein are therefore
35 to be construed accordingly, with such uses and effects

employing modified or equivalent factors as aforesaid being part of the invention.

The peptides useful in the invention can be made recombinantly using DNA constructs comprising DNA sequences, as defined above, in operable reading frame position in vectors under the control of control sequences so as to permit expression of the sequences in suitable host cells after transformation thereof by said constructs (preferably the control sequence includes a regulatable promoter, e.g. Trp) - it will be appreciated that the selection of a promoter and regulatory sequences (if any) are matters of choice for those of ordinary skill in the art.

The factors of the invention can be formulated for pharmaceutical or veterinary use by combination with an acceptable diluent, carrier or excipient and/or in unit dosage form. In using the factors of the invention, conventional pharmaceutical or veterinary practice may be employed to provide suitable formulations or compositions.

Thus, the formulations of this invention can be applied to parenteral administration, for example, intravenous, subcutaneous, intramuscular, intraorbital, opthalmic, intraventricular, intracranial, intracapsular, intraspinal, intracisternal, intraperitoneal, topical, intranasal, aerosol, scarification, and also oral, buccal, rectal or vaginal administration.

The formulations of the invention may also be administered by the transplantation into the patient of host cells expressing the DNA of the instant invention or by the use of surgical implants which release the formulations of the invention.

Parenteral formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal

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drops, or aerosols.

Methods well known in the art for making formulations are to be found in, for example, "Remington's Pharmaceutical Sciences." Formulations for parenteral
5 administration may, for example, contain as excipients sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes, biocompatible, biodegradable lactide polymer, or polyoxyethylene-polyoxypropylene
10 copolymers may be used to control the release of the present factors. Other potentially useful parenteral delivery systems for the factors include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for
15 inhalation may contain as excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel to be applied
20 intranasally. Formulations for parenteral administration may also include glycocholate for buccal administration, methoxysalicylate for rectal administration, or citric acid for vaginal administration.

The present factors can be used as the sole active
25 agents or can be used in combination with other active ingredients.

The concentration of the present factors in the formulations of the invention will vary depending upon a number of issues, including the dosage to be administered,
30 and the route of administration.

In general terms, the factors of this invention may be provided in an aqueous physiological buffer solution containing about 0.1 to 10% w/v compound for parenteral administration. General dose ranges are from about 1 μg/kg
35 to about 1 g/kg of body weight per day; a preferred dose

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range is from about 0.01 mg/kg to 100 mg/kg of body weight per day. The preferred dosage to be administered is likely to depend upon the type and extent of progression of the pathophysiological condition being addressed, the overall
5 health of the patient, the make up of the formulation, and the route of administration.

As indicated above, cell proliferation, particularly that of Schwann cells (the glial cells of the peripheral nervous system) and other cells of the nervous system is
10 inhibited in the presence of the factors of the invention.

There are a variety of tumors of glial cells, the most common of which is probably neurofibromatosis, which is a patchy small tumor created by overgrowth of glial cells. Also, it has been found that an activity very much
15 like GGF can be found in some Schwann cell tumors (Brockes et al., Ann. Neurol. 20:317 (1986)). Therefore inhibitors of GGF action on their receptors provides a therapy of a glial tumor. This therapy comprises administering an effective amount of a substance which inhibits the binding
20 of a stimulatory factor as defined above to its receptor. Additionally, given the association of GGF receptor amplification with human adenocarcinomas (Kraus et al., (1987) EMBO J. 6:605; Slamon et al. (1987) Science 235:177; Varley et al. (1987) Oncogene 1:423; and van de Vijver et
25 al. (1987) Mol Cell Biol 7:2019) and tumors of the breast and ovary (Slamon et al. supra; Varley et al. supra: Venter et al. (1987) Lancet ii:67; Zhou et al. (1987) Cancer Res. 47:6123; Berger et al. (1988) Cancer Res. 48:1238; Tsuda et al. (1989) Cancer Res. 49:3104; Slamon et al. (1989)
30 Science 244:707), a similar therapeutic approach may be taken with adenocarcinomas and tumors of breast and ovarian tissues.

In general, the invention includes the use of present polypeptide factors in the prophylaxis or treatment
35 of any pathophysiological condition in which a

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factor-sensitive or factor-responsive cell type is
involved.

     The polypeptide factors of the invention can also be
used as immunogens for making antibodies, such as
5 monoclonal antibodies, following standard techniques. Such
antibodies are included within the present invention.
These antibodies can be used for therapeutic or diagnostic
purposes. Thus, conditions associated with abnormal levels
of the factor may be tracked by using such antibodies. In
10 vitro techniques can be used, employing assays on isolated
samples using standard methods. Imaging methods can also
be employed in which the antibodies are, for example,
tagged with radioactive isotopes which can be remotely
imaged from outside the body using techniques employed in
15 the art of, for example, tumour imaging.

     Such antibodies, as described above, may also be
used for therapeutic purposes. Anti-idiotypic antibodies
raised against the polypeptide factors of the invention or
idiotypic antibodies raised against their cognate receptor
20 can be used as antagonists of GGF/erbB2 ligand induced
proliferation of p185^{erbB2} bearing cells.

     The invention also includes the general use of the
present factors as inhibitors of cell proliferation in vivo
or in vitro, and the methods for such use. One embodiment
25 is thus a method for producing a tumor cell
antiproliferative effect in a vertebrate by administering
an effective amount of a factor of the invention. An
example of such a method is the treatment or prophylaxis of
nervous system tumors or tumors of other tissues.

30   A further general aspect of the invention is the use
of a factor of the invention in the manufacture of a
medicament, preferably for the treatment of a nervous
disease or disorder.

     Also included in the invention are the use of the
35 factors of the invention in competitive assays to identify

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or quantify molecules having receptor binding characteristics corresponding to those of said polypeptides. The polypeptides may be labelled, optionally with a radioisotope and these labelled products may be used
5 to determine if receptor binding exists. A competition assay can identify both antagonists and agonists of the relevant receptor. Any competition for receptor binding between a known agonist and an antagonist (shown to bind the receptor) in a bioassay would be reflected by a
10 decrease in biological activity with in an increase in concentration of antagonist.

In another aspect, the invention provides the use of the factors in an affinity isolation process, e.g., affinity chromatography, for the separation of a respective
15 corresponding receptor. Such processes for the isolation of receptors corresponding to particular proteins are known in the art, and a number of techniques are available and can be applied to the factors of the present invention. For example, in relation to IL-6 and IFN-gamma, the reader
20 is referred to Novick et al. ((1990) J. Chromatogr. 510:331-7), in relation to gonadotropin releasing hormone, reference is made to Hazum ((1990) J. Chromatogr. 510:233-8), in relation to G-CSF, reference is made to Fukunaga et al. ((1990) J. Biol. Chem. 265:13386-13390), in
25 relation to IL-2, reference is made to Smart et al. ((1990) J. Invest. Dermatol. 94:158S-163S), and in relation to human IFN-gamma, reference is made to Stefanos et al. ((1989) J. Interferon Res. 9:719-30)

The following examples are not intended to limit the
30 invention, but are intended to usefully illustrate the same, and provide specific guidance for effective preparative techniques. Examples 1-4 teach the purification and consequent cloning of bovine DNA sequences encoding GGF Examples 5 and 7 demonstrate the isolation of
35 human DNA sequences encoding GGF. Examples 8 and 9

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demonstrate the isolation of splicing variants. Examples 10 and 11 show specific antiproliferative variants and examples of their function. Examples 12 and 13 demonstrate the production and testing of antiproliferative molecules.

5   Brief Description of the Drawings
           The drawings will first be described.
Drawings
           Figures 1 to 8 relate to Example 1 hereinafter, and are briefly described below:
10   Figure 1 is the profile for product from carboxymethyl cellulose chromatography;
           Figure 2 is the profile for product from hydroxylapatite HPLC;
           Figure 3 is the profile for product from Mono S
15 FPLC;
           Figure 4 is the profile for product from Gel filtration FPLC;
           Figures 5 and 6 depict the profiles for the two partially purified polypeptide products from reversed-phase
20 HPLC; and
           Figures 7 and 8 depict dose response curves for the GGF-I and GGF-II fractions from reversed-phase HPLC using either a fetal calf serum or a fetal calf plasma background;
25   Figures 9 to 12 depict peptides derived from GGF-I and GGF-II, (SEQ ID Nos. 1-53 and 169) (see Example 2, hereinafter), Figures 10 and 12 specifically depict novel sequences:
           In Figure 10, Panel A, the sequences of GGF-I
30 peptides used to design degenerate oligonucleotide probes and degenerate PCR primers are listed (SEQ ID Nos. 20-30). Some of those sequences in Panel A were also used to design synthetic peptides. Panel B shows the novel peptides that were too short (less than 6 amino acids) for the design of

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degenerate probes or degenerate PCR primers (SEQ ID Nos. 31 and 52);

In Figure 12, Panel A, the sequences of GGF-II peptides used to design degenerate oligonucleotide probes
5 and degenerate PCR primers are listed (SEQ ID Nos. 45-52). Some of these sequences in Panel A were also used to design synthetic peptides. Panel B shows the novel peptides that were too short (less than 6 amino acids) for the design of degenerate probes or degenerate PCR primers (SEQ ID No.
10 53);

Figures 13 to 20 relate to Example 3 hereinafter, and show various aspects of the mitogenic activity of factors related to the invention;

Figures 21 to 28 (a, b and c) relate to Example 4
15 hereinafter, and are briefly described below:

Figure 21 lists the degenerate oligonucleotide probes (SEQ ID Nos. 54-88) designed from the novel peptide sequences listed in Figure 10, Panel A and Figure 12, Panel A;

20 Figure 22 (SEQ ID No. 89) depicts a stretch of the putative bovine GGF-II gene sequence from the recombinant bovine genomic phage GGF2BG1, which contains the binding site of degenerate oliognucleotide probes 609 and 650 (see Figure 21, SEQ ID Nos. 69 and 72, respectively).

25 Shown are the coding strand of the DNA sequence and the deduced amino acid sequence in the third reading frame. The sequence of peptide 12 from GGF-2 (shown in bold) is part of a 66 amino acid open reading frame (nucleotides 75272);

30 Figure 23 lists the degenerate PCR primers (Panel A, SEQ ID No. 90-108) and unique PCR primers (Panel B, SEQ ID Nos. 109-119) used in experiments to isolate segments of the bovine GGF-II coding sequences present in RNA from posterior pituitary;

Figure 24 summarizes the nine distinct contiguous
bovine GGF-II cDNA structures and sequences that were
obtained in PCR amplification experiments using the list of
primers in Figure 7, Panels A and B, on RNA from posterior
5   pituitary. The top line of the Figure shows a schematic of
the exon sequences which contribute to the cDNA structures
that were characterized;

Figure 25 is a physical map of bovine recombinant
phage GGF2BG1. The bovine DNA fragment is roughly 20 kb in
10 length and contains two exons (bold) of the bovine GGFII
gene. Restriction sites for the enzymes Xbal, Spe I, Ndel,
EcoRI, Kpnl, and SstI have been placed on this physical
map.   Shaded portions correspond to fragments which were
subcloned for sequencing;

15   Figure 26 shows schematically the structure of three
alternative gene products of the putative bovine GGF-II
gene. Exons are listed A through E in the order of their
discovery. The alternative splicing patterns 1, 2 and 3
generate three overlapping deduced protein structures
20 (GGF2BPP1, 2, and 3), which are displayed in the various
Figures 28;

Figure 27 compares the GGF-I and GGF-II sequences
identified in the deduced protein sequences (SEQ ID Nos.
120-132) shown in Figures 28a, 28b and 28c with the novel
25 peptide sequences listed in Figures 10 and 12. The Figure
shows that six of the nine novel GGF-II peptide sequences
are accounted for in these deduced protein sequences. Two
peptide sequences similar to GGF-I sequences are also
found;

30   Figure 28a shows the coding strand DNA sequence and
deduced amino acid sequence of the cDNA (SEQ ID No. 133)
obtained from splicing pattern number 1 shown in Figure 26.
This partial cDNA of the putative bovine GGF-II gene
encodes a protein of 206 amino acids in length. Peptides
35 shown in bold were those identified from the lists

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presented in Figures 10 and 12. Potential glycosylation
sites are underlined (along with polyadenylation signal
AATAAA);

Figure 28b shows the coding strand DNA sequence and
5   deduced amino acid sequence of the cDNA (SEQ ID No. 134)
obtained from splicing pattern number 2 shown in Figure 26.
This partial cDNA of the putative bovine GGF-II gene
encodes a protein of 281 amino acids in length. Peptides
shown in bold were those identified from the lists
10 presented in Figures 10 and 12. Potential glycosylation
sites are underlined (along with polyadenylation signal
AATAAA);

Figure 28c shows the coding strand DNA sequence and
deduced amino acid sequence of the cDNA (SEQ ID No. 135)
15 obtained from splicing pattern number 3 shown in Figure 26.
This partial cDNA of the putative bovine GGF-II gene
encodes a protein of 257 amino acids in length. Peptides
shown in bold were those identified from the lists
presented in Figures 10 and 12. Potential glycosylation
20 sites are underlined (along with polyadenylation signal
AATAAA); and the DNA sequences shown in Figures 28a, 28b
and 28c are themselves further aspects of this invention;
and the invention further includes polypeptides encoded by
said sequences;

25   Figure 29 relates to Example 7, hereinafter, and
shows an autoradiogram of a cross hybridization analysis of
putative bovine GGF-II gene sequences to a variety of
mammalian DNAs on a Southern blot. The filter contains
lanes of Eco RI-digested DNA (5 Mg per lane) from the
30 species listed in the Figure. The probe detects a single
strong band in each DNA sample, including a four kb
fragment in the bovine DNA as anticipated by the physical
map in Figure 25. Bands of relatively minor intensity are
observed as well, which could represent related DNA
35 sequences. The strong hybridizing band from each of the

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other mammalian DNA samples presumably represents the
GGF-II homologue of those species.

In Example 1 hereinafter, unless otherwise
indicated, all operations were conducted at 40°C, and, with
5 reference to Figures 1 to 6, activity at each stage was
determined using the Brockes (Meth. Enz., supra) techniques
with the following modifications. Thus, in preparing
Schwann cells, 5μM forskolin was added in addition to DMEM
(Dulbecco's modified Eagle's medium), FCS and GGF. Cells
10 used in the assay were fibroblast-free Schwann cells at
passage number less than 10, and these cells were removed
from flasks with trypsin and plated into flat-bottomed
96-well plates at 3.3 thousand cells per microwell.

^{125}IIUdR was added for the final 24 hours after the
15 test solution addition. The background (unstimulated)
incorporation to each assay was less than 100 cpm, and
maximal incorporation was 20 to 200 fold over background
depending on Schwann cell batch and passage number.

In the case of the GGF-I and GGF-II fractions from
20 reversed-phase HPLC as described below in Example 1, two
dose response curves were also produced for each factor,
using exactly the above method for one of the curves for
each factor, and the above method modified in the assay
procedure only by substituting fetal calf plasma for fetal
25 calf serum to obtain the other curve for each factor. The
results are in Figures 7 and 8.

Figure 30 is a schematic diagram of representative
splicing variants. The coding segments are represented by
F, E, B, A, G, C, C/D, C/D', D, D', H, K and L. The
30 location of the peptide sequences derived from purified
protein are indicated by "o".

Figure 31 (SEQ ID No. 136-147, 160, 161 and 168) (is
a listing of the DNA sequences and predicted peptide
sequences of the coding segments of GGF. Line 1 represents
35 the predicted amino acid sequence of bovine GGF, line 2

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represents the nucleotide sequence of bovine GGF, line 3
represents the nucleotide sequence of human GGF (heregulin)
(nucleotide base matches are indicated with a vertical
line) and line 4 represents the predicted amino acid
5 sequence of human GGF/heregulin *where it differs from the*
predicted bovine sequence. Coding segment K represents
only the bovine sequence. The human and bovine coding
segments for both E and A' are provided. Coding segment D'
represents only the human (heregulin) sequence.

10   Figure 32 is the predicted GGF2 amino acid sequence
and nucleotide sequence of BPP5 (SEQ ID No. 148). The
upper line represents the nucleotide sequence and the lower
line represents the predicted amino acid sequence.

          Figure 33 is the predicted amino acid sequence and
15 nucleotide sequence of GGF2BPP2 (SEQ ID No. 149). The
upper line represents the nucleotide sequence and the lower
line represents the predicted amino acid sequence.

          Figure 34 is the predicted amino acid sequence and
nucleotide sequence of GGF2BPP4. The upper line represents
20 the nucleotide sequence and the lower line represents the
predicted amino acid sequence.

          Figure 35 (SEQ ID Nos. 151-152) depicts the
alignment of two GGF peptide sequences (GGF2bpp4 and
GGF2bpp5) with the human EGF (hEGF) peptides sequences.
25 Asterisks indicate positions of conserved cysteines.

          Figure 36 depicts the level of GGF activity (Schwann
cell mitogenic assay) and tyrosine phosphorylation of a ca.
200kD protein (intensity of a 200 kD band on an
autoradiogram of a Western blot developed with an
30 antiphosphotyrosine polyclonal antibody) in response to
increasing amounts of GGF.

          Figure 37 is a list of splicing variants derived
from the sequences shown in Figure 31.

          Figure 38 is a scale coding segment map of the
35 clone.   T3 refers to the bacteriophage promoter used to

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produce mRNA from the clone.   R = flanking EcoRI
restriction enzyme sites.   5' UT refers to the 5'
untranslated region. E, B, A, C, C/D', and D refer to the
coding segments. O = the translation start site. ∧ = the
5 5' limit of the region homologous to the bovine E segment
(see example 6) and 3' UT refers to the 3' untranslated
region.

Figure 39 is the predicted amino acid sequence,
bottom, and nucleic sequence, top, of EGFL1 (SEQ ID No.
10 154).

Figure 40 is the predicted amino acid sequence,
bottom, and nucleic sequence, top, of EGFL2 (SEQ ID No.
155).

Figure 41 is the predicted amino acid sequence,
15 bottom, and nucleic sequence, top, of EGFL3 (SEQ ID No.
156).

Figure 42 is the predicted amino acid sequence,
bottom, and nucleic sequence, top, of EGFL4 (SEQ ID No.
157).

20   Figure 43 is the predicted amino acid sequence,
bottom, and nucleic sequence, top, of EGFL5 (SEQ ID No.
158).

Figure 44 is the predicted amino acid sequence,
bottom, and nucleic sequence, top, of EGFL6 (SEQ ID No.
25 159).

Figure 45 is the predicted amino acid sequence
(middle) and nucleic sequence (top) of GGF2HBS5 (SEQ ID No.
167). The bottom (intermittent sequence represents peptide
sequences derived from GGFII preparations (see Figures 11,
30 12).

Detailed Description

The invention pertains to methods for the use of
novel factors which are inhibitors of cell, particularly
neural and glial cell proliferation, and the use of DNA

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sequences encoding these factors. Disclosed are several
gene splicing variants of these factors which may encode
inhibitors of cell division.

5   Holmes et al. ((1992) Science 256:1205) and Wen et
al. ((1992) Cell 69:559) demonstrate that DNA sequences
encoding proteins which bind to a receptor associated with
several human tumors (p185^{erbB2}) share a great deal of
homology with GGF DNA sequences. This provides evidence to
indicate that the bovine GGFs and the human and rat
10 p185^{erbB2} ligands are encoded by the same (homologous) gene
and that ligand groups both interact with the same receptor
(p185^{erbB2}).

      The p185^{erbB2} protein is a 185 kilodalton membrane
spanning protein with tyrosine kinase activity. The
15 protein is encoded by the erbB2 proto-oncogene (Yarden and
Ullrich. (1988) Ann. Rev. Biochem. 57:443). The erbB2
gene, also referred to as HER-2 (in human cells) and neu
(in rat cells), is closely related to the receptor for
epidermal growth factor (EGF). Recent evidence indicates
20 that proteins which interact with (and activate the kinase
of) p185^{erbB2} induce proliferation in the cells bearing
p185^{erbB2} (Holmes et al. (1992) Science 256:1205; Dobashi et
al. (1991) Proc. Natl. Acad. Sci. 88:8582; Lupu et al.
(1992) Proc. Natl. Acad. Sci. 89:2287). This evidence
25 supports the conclusion that the gene encoding GGF's and
the p185^{erbB2} binding proteins are responsible for the
production of a family of growth factors which have
pleiotrophic effects in that they target both neural cells,
particularly Schwann cells, and cells which give rise to
30 human adenocarcinoma and other carcinomas.

      Furthermore, it is evident that the gene encoding
GGF and p185^{erbB2} binding proteins produces a number of
variably-sized, differentially-spliced RNA transcripts that
give rise to a series of proteins, which are of different
35 lengths and contain some common peptide sequences and some

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unique peptide sequences. This is supported by the
evidence that differentially spliced sequences are
recoverable from bovine posterior pituitary RNA (as
presented herein), and human breast cancer cell line
5   (MDA-MB-231) RNA (Holmes et al. (1992) Science 256:1205).
Further support for this "one gene: multiple product"
conclusion derives from the wide size range of proteins
which act as both mitogens for Schwann cells (as disclosed
herein) and ligands for the $p185^{erbB2}$ receptor (see below).

10   Further evidence to support the fact that the genes
encoding GGF and $p185^{erbB2}$ receptor ligands are homologous
comes from nucleotide sequence comparison. Holmes et al.
((1992) Science, 256:1205-1210) demonstrate the
purification of a 45-kilodalton human protein (heregulin)
15 which specifically interacts with the $p185^{erbB2}$ receptor.
The predicted sequences of the polypeptides encoded by
these human DNA sequences match very closely with the
sequences predicted from the Glial Growth Factor sequences.
Peles et al. ((1992) Cell 69:205) and Wen et al ((1992)
20 Cell 69:559) describe a complementary DNA isolated from rat
cells encoding a protein called *neu* differentiation factor
(NDF), which shares homology with the heregulin sequences
described by Holmes et al. In addition, the translation
product of the NDF cDNA has $p185^{erbB2}$ binding activity.
25 Several other groups have reported the purification of
proteins of various molecular weights with *erbB2* binding
activity. These groups include Lupu et al. ((1992) Proc.
Natl. Acad. Sci. USA 89:2287), Yarden and Peles ((1991)
Biochemistry 30:3543), Lupu et al ((1990) Science
30 249:1552), and Dobashi et al. ((1991) Biochem Biophys. Res.
Comm. 179:1536).

         It has been established that the $p185^{erbB2}$ oncogene
and, by inference, its cognate ligands play a significant
role in the development and maintenance of several types of
35 tumors. Amplification and overexpression of *erbB2* has been

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associated with human adenocarcinomas from several tissues
(Kraus et al. (1987) EMBO J. 6:605; Slamon et al. (1987)
Science 235:177; Varley et al. (1987) Oncogene 1:423; and
van de Vijver et al. (1987) Mol Cell Biol 7:2019). An
5 association has also been reported with breast and ovarian
cancer (Slamon et al. supra; Varley et al. supra: Venter et
al. (1987) Lancet ii:67; Zhou et al. (1987) Cancer Res.
47:6123; Berger et al. (1988) Cancer Res. 48:1238; Tsuda et
al. (1989) Cancer Res. 49:3104; Slamon et al. (1989)
10 Science 244:707).

There is also evidence that the *erbB2* gene plays a
role in oncogenesis of cells of the Schwann cell lineage
(Perantoni et al. (1987) Proc. Nat. Acad. Sci. 84:6317;
Nikitin et al. (1991) Proc. Nat. Acad. Sci. 88:9939).
15 Several tumor types are a result of abnormal proliferation
of Schwann cells and these include neurofibromas, and
malignant schwannomas and neurofibrosarcomas.

As candidate ligands for the *erbB2* receptor, the
GGFs could play a significant role in the development of
20 the tumors described above.

As outlined above, the gene encoding the GGFs and
the p185^{erbB2} ligands gives rise to a number of variant
transcripts which encode a variety of proteins. Several of
these variant proteins bind to the p185^{erbB2} receptor on
25 neural cells, including Schwann cells (described above and
disclosed herein), as well as to the same receptor on tumor
cell lines as described above. Some of these variant
proteins activate cell proliferation in Schwann cells and
in tumor cell lines (described above and disclosed herein).
30 Other variants may possibly interfere with the activity of
the ligands which stimulate proliferation by competing
with those ligands for binding sites on the p185^{erbB2}
receptor. Chan et al. ((1991) Science 254:1382) showed
that a naturally occurring hepatocyte growth factor (HGF)
35 variant was derived from a smaller transcript encoded by

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the same gene as the full length molecule. The truncated protein encoded by the variant transcript specifically inhibits HGF induced mitogenesis and was demonstrated to compete with HGF for binding to the HGF receptor. The HGF
5 receptor has been identified as the *c-met* proto-oncogene product. Thus, these variant versions of growth factor proteins may play a significant regulatory role in the control of cell proliferation. GGF-related factors which inhibit glial proliferation will be therapeutically useful
10 as anti-proliferative compounds for the treatment of tumors of the neural system.

It has been shown that myelination by Schwann cells and oligodendrocytes is regulated by the proliferative state (Jessen et al., 1991 *Ann NY Acad Science* 633:78-89).
15 When the cell withdraws from the proliferative cycle the myelination process appears to begin. Factors of the invention which induce Schwann cells and oligodendrocytes to exit the proliferative cell cycle and enter the quiescent state may be administered to increase myelination
20 of existing or newly regenerated neural tissue in a mammal suffering from diseases or disorders of demyelination. Examples of diseases and disorders which may be treated using an inhibitor of mutagenesis include Charot-Marie-Tooth disease (particularly type I and type III), peroneal
25 muscular atrophy, Dejérine-Sottos disease (type III hereditary motor and sensory neuropathy), multiple sclerosis, chronic inflammatory demyelinating polyradiculoneuropathy, chronic liver disease, diphtheritic polyneuritis, Guillain-Barré syndrome, hypothyroid
30 polyneuropathy, metachromatic leukodystrophy, type I hereditary motor and sensory neuropathy, type III hereditary motor and sensory neuropathy, and vasculitic neuropathy.

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EXAMPLE 1

I.   Preparation of Factor-CM Fraction

4,000 frozen whole bovine pituitaries (c.a. 12 kg) were thawed overnight, washed briefly with water and then
5   homogenized in an equal volume of 0.15 M ammonium sulphate in batches in a Waring Blender. The homogenate was taken to pH 4.5 with 1.0 M HCl and centrifuged at 4,900g for 80 minutes. Any fatty material in the supernatant was removed by passing it through glass wool. After taking the pH of
10 the supernatant to 6.5 using 1.0 M NaOH, solid ammonium sulphate was added to give a 36% saturated solution. After several hours stirring, the suspension was centrifuged at 4,900 g for 80 minutes and the precipitate discarded. After filtration through glass wool, further solid ammonium
15 sulphate was added to the supernatant to give a 75% saturated solution which was once again centrifuged at 4,900 g for 80 minutes after several hours stirring. The pellet was resuspended in c.a. 2 L of 0.1 M sodium phosphate pH 6.0 and dialyzed 3 x 40 L of the same buffer.
20 After confirming that the conductivity of the dialysate was below 20.0 μSiemens, it was loaded onto a Bioprocess column (120 x 113 mm, Pharmacia) packed with carboxymethyl cellulose (CM-52, Whatman) at a flow rate of 2 μl.min^{-1} The column was washed with 2 volumes of 0.1 M sodium
25 phosphate pH 6.0, followed by 2 volumes of 50 mM NaCl, and finally 2 volumes of 0.2 M NaCl both in the same buffer. During the final step, 10 μL (5 minute) fractions were collected. Fractions 73 to 118 inclusive were pooled, dialyzed against the 10 volumes of 10 mM sodium phosphate
30 pH 6.0 twice and clarified by centrifugation at 100,000 g for 60 minutes.

II.   Hydroxylapatite HPLC

Hydroxylapatite HPLC is not a technique hitherto used in isolating glial growth factors, but proved
35 particularly efficacious in this invention.

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The material obtained from the above CM-cellulose chromatography was filtered through a 0.22 μm filter (Nalgene), loaded at room temperature on to a high performance hydroxylapatite column (50 x 50 mm, Biorad) equipped with a guard column (15 x 25 mm, Biorad) and equilibrated with 10 mM potassium phosphate pH 6.0. Elution at room temperature was carried out at a flow rate of 2 μl.minute^{-1} using the following programmed linear gradient:

time (min)	%B	Solvent A :	10 mM potassium phosphate pH 6.0
0.0	0	Solvent B :	1.0 M potassium phosphate pH 6.0
5.0	0		
7.0	20		
70.0	20		
150.0	100		
180.0	100		
185.0	0		

6.0 μL (3 minutes) fractions were collected during the gradient elution. Fractions 39-45 were pooled and dialyzed against 10 volumes of 50 mM sodium phosphate pH 6.0.

III. Mono S FPLC

Mono S FPLC enabled a more concentrated material to be prepared for subsequent gel filtration.

Any particulate material in the pooled material from the hydroxylapatite column was removed by a clarifying spin at 100,000 g for 60 minutes prior to loading on to a preparative HR10/10 Mono S cation exchange column (100 x 10 mm, Pharmacia) which was then re-equilibrated to 50 MM sodium phosphate pH 6.0 at room temperature with a flow rate of 1.0 μL.minute^{-1}. Under these conditions, bound protein was eluted using the following programmed linear gradient:

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time (min)	%B	Solvent A :	50 mM potassium phosphate
pH 6. 0			
0.0	0	Solvent B :	1.2 M sodium chloride, 50
mm			
5 70.0	30		sodium phosphate pH
6.0			
240.0	100		
250.0	100		
260.0	0		

10   1 μL (1 minute) fractions were collected throughout this gradient program. Fractions 99 to 115 inclusive were pooled.

IV.   Gel Filtration FPLC

This step commenced the separation of the two
15 factors of the invention prior to final purification, producing enriched fractions.

For the purposes of this step, a preparative Superose 12 FPLC column (510 x 20 mm, Pharmacia) was packed according to the manufacturers' instructions. In order to
20 standardize this column, a theoretical plates measurement was made according to the manufacturers' instructions, giving a value of 9,700 theoretical plates.

The pool of Mono S eluted material was applied at room temperature in 2.5 μL aliquots to this column in 50 MM
25 sodium phosphate, 0.75 NaCl pH 6.0 (previously passed through a C18 reversed phase column (Sep-pak, Millipore) at a flow rate of 1.0 μL.minute^{-1}. 1 μL (0.5 minute) fractions were collected from 35 minutes after each sample was applied to the column. Fractions 27 to 41 (GGF-II) and 42
30 to 57 (GGF-I) inclusive from each run were pooled.

V.   Reversed-Phase HPLC

The GGF-I and GGF-II pools from the above Superose

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12 runs were each divided into three equal aliquots. Each aliquot was loaded on to a C8 reversed-phase column (Aquapore RP-300 7 μ C8 220 x 4.6 mm, Applied Biosystems) protected by a guard cartridge (RP-8, 15 x 3.2 mm, Applied
5 Biosystems) and equilibrated to 4°C at 0.5 μL.minute. Protein was eluted under these conditions using the following programmed linear gradient:

time (min) (TFA)	%B	Solvent A :	0.1% trifluoroacetic acid
10 0		Solvent B :	90% acetonitrile, 0.1% TFA
60	66.6		
62.0	100		
72.0	100		
75.0	0		

15 200 μL (0.4 minute) fractions were collected in siliconized tubes (Multilube tubes, Bioquote) from 15.2 minutes after the Beginning of the programmed gradient.

VI.   SDS-Polyacrylamide Gel Electrophoresis

        In this step, protein molecular weight standards,
20 low   range,   catalogue   no.   161-0304,   from   Bio-Rad Laboratories Limited, Watford, England were employed. The actual proteins used, and their molecular weight standards, have been listed hereinbefore.

        Fractions 47 to 53 (GGF-I) and fractions 61 to 67
25 (GGF-II) from the reversed-phase runs were individually pooled. 7 μL of the pooled material was boiled in an equal volume of 0.0125 M Tris-Cl, 4% SDS, 20% glycerol, and 10% ß-mercaptoethanol for GGF-I, for 5 minutes and loaded on to an 11% polyacrylamide Leammli gel with a 4% stacking gel
30 and run at a constant voltage of 50 V for 16 hours. This gel was then fixed and stained using a silver staining kit (Amersham).   Under these conditions, the factors are each

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seen as a somewhat diffuse band at relative molecular
weights 30,000 to 36,000 Daltons (GGF-I) and 55,000 to
63,000 Daltons (GGF-II), as defined by molecular weight
markers. From the gel staining, it is apparent that there
5 are a small number of other protein species present at
equivalent levels to the GGF-I and GGF-II species in the
material pooled from the reversed-phase runs.

VII. Stability in Trifluoroacetic Acid
        Stability data were obtained for the present Factors
10 in the presence of trifluoroacetic acid, as follows:
GGF-I
        Material from the reversed-phase HPLC, in the
presence of 0.1% TFA and acetonitrile, was assayed within
12 hours of the completion of the column run and then after
15 10 weeks incubation at 40˚C. Following incubation, the
GGF-I had at least 50% of the activity of that material
assayed directly off the column.
GGF-II
        Material from the reversed-phase HPLC, in the
20 presence of 0.1% TFA and acetonitrile, and stored at -20°C,
was assayed after thawing and then after 4 days incubation
at 40°C. Following incubation, the GGF-II had at least 50%
of the activity of that material freshly thawed.
        It will be appreciated that the trifluoroacetic acid
25 concentration used in the above studies is that most
commonly used for reversed-phase chromatography.

EXAMPLE 2
Amino acid sequences purified GGF-I and GGF-II
        Amino acid sequence analysis studies were performed
30 using highly purified bovine pituitary GGF-I and GGF-II.
The conventional single letter code was used to describe
the sequences. Peptides were obtained by lysyl
endopeptidase and protease V8 digests, carried out on

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reduced and carboxymethylated samples, with the lysyl endopeptidase digest of GGF-II carried out on material eluted from the 55-65 RD region of a 11% SDS-PAGE (MW relative to the above-quoted markers).

5   A total of 21 peptide sequences (see Figure 9) were obtained for GGF-I, of which 12 peptides (see Figure 10) are not present in current protein databases and therefore represent unique-sequences. A total of 12 peptide sequences (see Figure 11) were obtained for GGF-II, of

10   which 10 peptides (see Figure 12) are not present in current protein databases and therefore represent unique sequences (an exception is peptide GGF-II 06 (SEQ ID No. 38) which shows identical sequences in many proteins which are probably of no significance given the small number of

15   residues). These novel sequences are extremely likely to correspond to portions of the true amino acid sequences of GGFs I and II.

        Particular attention can be drawn to the sequences of GGF-I 07 (SEQ ID No. 39) and GGF-II 12 (SEQ ID No. 44),

20   which are clearly highly related. The similarities indicate that the sequences of these peptides are almost certainly those of the assigned GGF species, and are most unlikely to be derived from contaminant proteins.

        In addition, in peptide GGF-II 02 (SEQ ID No. 34),

25   the sequence X S S is consistent with the presence of an N linked carbohydrate moiety on an asparagine at the position denoted by X.

        In general, in Figures 9 and 11, X represents an unknown residue denoting a sequencing cycle where a single

30   position could not be called with certainty either because there was more than one signal of equal size in the cycle or because no signal was present. As asterisk denotes those peptides where the last amino acid called corresponds to the last amino acid present in that peptide. In the

35   remaining peptides, the signal strength after the last

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amino acid called was insufficient to continue sequence
calling to the end of that peptide. The right hand column
indicates the results of a computer database search using
the GCG package FASTA and TFASTA programs to analyze the
5   NBRF and EMBL sequence databases. The name of a protein in
this column denotes identity of a portion of its sequence
with the peptide amino acid sequence called allowing a
maximum of two mismatches. A question mark denotes three
mismatches allowed. The abbreviations used are as follows:

10 HMG-1   High Mobility Group protein-1
    HMG-2   High Mobility Group protein-2
    LH-alpha   Luteinizing hormone alpha subunit
    LH-beta   Luteinizing hormone beta subunit

EXAMPLE 3

15   Mitogenic activity of purified GGF-I and GGF-II
         The mitogenic activity of a highly purified sample
containing both GGFs I and II was studied using a
quantative method, which allows a single microculture to be
examined for DNA synthesis, cell morphology, cell number
20 and expression of cell antigens. This technique has been
modified from a method previously reported by Muir et al.
((1990) Analytical Biochemistry 185:377-382). The main
modifications are: 1) the use of uncoated microtiter
plates, 2) the cell number per well, 3) the use of 5% Fetal
25 Bovine Plasma (FBP) instead of 10% Fetal Calf Serum (FCS),
and 4) the time of incubation in presence of mitogens and
bromodeoxyuridine (BrdU), added simultaneously to the
cultures. In addition the cell monolayer was not washed
before fixation to avoid loss of cells, and the incubation
30 time of monoclonal mouse anti-BrdU antibody and peroxidase
conjugated goat anti-mouse immunoglobulin (IgG) antibody
were doubled to increase the sensitivity of the assay. The
assay, optimized for rat sciatic nerve Schwann cells, has
also been used for several cell lines, after appropriate

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modifications to the cell culture conditions.

I. Methods of Mitogenesis Testing

       On day 1, purified Schwann cells were plated onto uncoated 96 well plates in 5% FBP/Dulbecco's Modified Eagle
5 Medium (DMEM) (5,000 cells/well). On day 2, GGFs or other test factors were added to the cultures, as well as BrdU at a final concentration of 10mm. After 48 hours (day 4) BrdU incorporation was terminated by aspirating the medium and cells were fixed with 200 μl/well of 70% ethanol for 20 min
10 at room temperature. Next, the cells were washed with water and the DNA denatured by incubation with 100 μl 2N HCl for 10 min at 37°C. Following aspiration, residual acid was neutralized by filling the wells with 0.1M borate buffer, pH 9.0, and the cells were washed with phosphate
15 buffered saline (PBS). Cells were then treated with 50 μl of blocking buffer (PBS containing 0.1% Triton X 100 and 2% normal goat serum) for 15 min at 37°C. After aspiration, monoclonal mouse anti-BrdU antibody (Dako Corp., Santa Barbara, CA) (50 μl/well, 1.4 mg/ml diluted in blocking
20 buffer) was added and incubated for two hours at 37°C. Unbound antibodies were removed by three washes in PBS containing 0.1% Triton X-100 and peroxidase-conjugated goat ant-mouse IgG antibody (Dako Corp., Santa Barbara, CA) (50 μl/well, 2 mg/ml diluted in blocking buffer) was added and
25 incubated for one hour at 37°C. After three washes in PBS/Triton and a final rinse in PBS, wells received 100 μl/well of 50 mM phosphate/citrate buffer, pH 5.0, containing 0.05% of the soluble chromogen o-phenylenediamine (OPD) and 0.02% H_2O_2. The reaction was
30 terminated after 5-20 min at room temperature, by pipetting 80 μl from each well to a clean plate containing 40 μl/well of 2N sulfuric acid. The absorbance was recorded at 490nm using a plate reader (Dynatech Labs). The assay plates containing the cell monolayers were washed twice with PBS

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and immunocytochemically stained for BrdU-DNA by adding 100 μl/well of the substrate diaminobenzidine (DAB) and 0.02% H_2O_2 to generate an insoluble product. After 10-20 min the staining reaction was stopped by washing with water, and

5   BrdU-positive nuclei observed and counted using an inverted microscope.   Occasionally,   negative   nuclei   were counterstained with 0-001% Toluidine blue and counted as before.

II.   Cell Lines used for Mitogenesis Assays

10   *Swiss 3T3 Fibroblasts*

Cells, from Flow Labs, were maintained in DMEM supplemented with 10% FCS, penicillin and streptomycin, at 37°C in a humidified atmosphere of 10% CO_2 in air.   Cells were fed or subcultured every two days.   For mitogenic

15   assay, cells were plated at a density of 5,000 cells/well in complete medium and incubated for a week until cells were confluent and quiescent. The serum containing medium was removed and the cell monolayer washed twice with serum free-medium.   100 μl of serum free medium containing

20   mitogens and 10μM BrdU were added to each well and incubated for 48 hours. Dose responses to GGFs and serum or PDGF (as a positive control) were performed.

BHK (Baby Hamster Kidney) 21 C13 Fibroblasts

Cells from European Collection of Animal Cell

25   Cultures (ECACC), were maintained in Glasgow Modified Eagle Medium (GMEM) supplemented with 5% tryptose phosphate broth, 5% FCS, penicillin and streptomycin, at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were fed or subcultured every two to three days. For mitogenic assay,

30   cells were plated at a density of 2,000 cell/well in complete medium for 24 hours. The serum containing medium was then removed and after washing with serum free medium, replaced with 100 μl of 0.1% FCS containing GMEM or GMEM

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alone.   GGFs and FCS or BFGF as positive controls were
added, coincident with 10μM BrdU, and incubated for 48
hours.   Cell cultures were then processed as described for
Schwann cells.

5 *C6 Rat Glioma Cell Line*
         Cells, obtained at passage 39, were maintained in
DMEM containing 5% FCS, 5% Horse serum (HS), penicillin and
streptomycin, at 37°C in a humidified atmosphere of 10% CO_2
in air.   Cells were fed or subcultured every three days.
10 For mitogenic assay, cells were plated at a density of
2,000 cells/well in complete medium and incubated for 24
hours.   Then medium was replaced with a mixture of 1:1 DMEM
and F12 medium containing 0.1% FCS, after washing in serum
free medium.   Dose responses to GGFs, FCS and AFGF were
15 then performed and cells were processed through the ELISA
as previously described for the other cell types.

PC12 (Rat Adrenal Pheochromocytoma Cells)
         Cells from ECACC, were maintained in RPMI 1640
supplemented with 10% HS,   5% FCS,   penicillin and
20 streptomycin,   in collagen coated flasks,   at 370C in a
humidified atmosphere of 5% CO_2 in air.   Cells were fed
every three days by replacing 80% of the medium.   For
mitogenic assay, cells were plated at a density of 3,000
cells/well in complete medium, on collagen coated plates
25 (50 μl/well collagen, Vitrogen Collagen Corp., diluted 1 :
50, 30 min at 37°C) and incubated for 24 hours.   The medium
was then placed with fresh RPMI either alone or containing
1 mM insulin or 1% FCS.   Dose responses to FCS/HS (1:2) as
positive control and to GGFs were performed as before.
30 After 48 hours cells were fixed and the ELISA performed as
previously described.

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III. Results of Mitogenesis Assays

All the experiments presented in this Example were performed using a highly purified sample from a Superose 12 chromatography purification step (see Example 1, section D)
5 containing a mixture of GGF-I and GGF-II (GGFs).

Firstly, the results obtained with the BrdU incorporation assay were compared with the classical mitogenic assay for Schwann cells based on ^{125}I-UdR incorporation into DNA of dividing cells, described by
10 J.P.Brockes ((1987) Methods Enzymol. 147:217).

Figure 13 shows the comparison of data obtained with the two assays, performed in the same cell culture conditions (5,000 cells/well, in 5% FBP/DMEM, incubated in presence of GGFs for 48hrs). As clearly shown, the results
15 are comparable, but BrdU incorporation assay appears to be slightly more sensitive, as suggested by the shift of the curve to the left of the graph, i.e. to lower concentrations of GGFS.

As described under the section "Methods", after the
20 immunoreactive BrdU-DNA has been quantitated by reading the intensity of the soluble product of the OPD peroxidase reaction, the original assay plates containing cell monolayers can undergo the second reaction resulting in the insoluble DAB product, which stains the BrdU positive
25 nuclei. The microcultures can then be examined under an inverted microscope, and cell morphology and the numbers of BrdU-positive and negative nuclei can be observed.

In Figure 14a and Figure 14b the BrdU-DNA immunoreactivity, evaluated by reading absorbance at 490
30 nm, is compared to the number of BrdU-positive nuclei and to the percentage of BrdU-positive nuclei on the total number of cells per well, counted in the same cultures. Standard deviations were less than 10%. The two evaluation methods show a very good correlation and the discrepancy
35 between the values at the highest dose of GGFs can be

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explained by the different extent of DNA synthesis in cells
detected as BrdU-positive.

The BrdU incorporation assay can therefore provide
additional useful information about the biological activity

5 of GGFs on Schwann cells when compared to the ^{125}I-UdR
incorporation assay. For example, the data reported in
Figure 15 show that GGFs can act on Schwann cells to induce
DNA synthesis, but at lower doses to increase the number of
negative cells present in the microculture after 48 hours.

10 The BrdU incorporation assay has been used on
several cell lines of different origin. In Figure 16 the
mitogenic responses of Schwann cells and Swiss 3T3
fibroblasts to GGFs are compared; despite the weak response
obtained in 3T3 fibroblasts, some clearly BrdU-positive

15 nuclei were detected in these cultures. Control cultures
were run in parallel in presence of several doses of FCS or
human recombinant PDGF, showing that the cells could
respond to appropriate stimuli (not shown).

The ability of fibroblasts to respond to GGFs was

20 further investigated using the BHK 21 C13 cell line. These
fibroblasts, derived from kidney, do not exhibit contact
inhibition or reach a quiescent state when confluent.
Therefore the experimental conditions were designed to have
a very low background proliferation without comprising the

25 cell viability. GGFs have a significant mitogenic activity
on BHK21 C13 cells as shown by Figure 17 and Figure 18.
Figure 17 shows the Brdu incorporation into DNA by BHK 21
C13 cells stimulated by GGFS in the presence of 0.1% FCS.
The good mitogenic response to FCS indicates that cell

30 culture conditions were not limiting. In Figure 18 the
mitogenic effect of GGFs is expressed as the number of
BrdU-positive and BrdU-negative cells and as the total
number of cells counted per well. Data are representative
of two experiments run in duplicates; at least three fields

35 per well were counted. As observed for Schwann cells in

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addition to a proliferative effect at low doses, GGFs also
increase the numbers of nonresponding cells surviving. The
percentage of BrdU positive cells is proportional to the
increasing amounts of GGFs added to the cultures. The
5   total number of cells after 48 hours in presence of higher
doses of GGFs is at least doubled, confirming that GGFs
induce DNA synthesis and proliferation in BHK21 C13 cells.
Under the same conditions, cells maintained for 48 hours in
the presence of 2% FCS showed an increase of about six fold
10   (not shown).

C6 glioma cells have provided a useful model to
study glial cell properties. The phenotype expressed seems
to be dependent on the cell passage, the cells more closely
resembling an astrocyte phenotype at an early stage, and an
15   oligodendrocyte phenotype at later stages (beyond passage
70). C6 cells used in these experiments were from passage
39 to passage 52. C6 cells are a highly proliferating
population, therefore the experimental conditions were
optimized to have a very low background of BrdU
20   incorporation. The presence of 0.1% serum was necessary to
maintain cell viability without significantly affecting the
mitogenic responses, as shown by the dose response to FCS
(Figure 19).

In Figure 20 the mitogenic responses to aFGF (acidic
25   Fibroblast growth factor) and GGFs are expressed as the
percentages of maximal BrdU incorporation obtained in the
presence of FCS (8%). Values are averages of two
experiments, run in duplicates. The effect of GGFs was
comparable to that of a pure preparation of aFGF. aFGF has
30   been described as a specific growth factor for C6 cells
(Lim R. et al. (1990) Cell Regulation 1:741-746) and for
that reason it was used as a positive control. The direct
counting of BrdU positive and negative cells was not
possible because of the high cell density in the
35   microcultures. In contrast to the cell lines so far

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reported, PC12 cells did not show any evident responsiveness to GGFS, when treated under culture conditions in which PC12 could respond to sera (mixture of FCS and HS as used routinely for cell maintenance).
5 Nevertheless the number of cells plated per well seems to affect the behavior of PC12 cells, and therefore further experiments are required.

EXAMPLE 4
Isolation and cloning of nucleotide sequences
10   encoding proteins containing GGF-I and GGF-II peptides
Isolation and cloning of the GGF-II nucleotide sequences was performed as outlined herein, using peptide sequence information and library screening, and was performed as set out below. It will be appreciated that
15 the peptides of Figures 4 and 5 can be used as the starting point for isolation and cloning of GGF-I sequences by following the techniques described herein. Indeed, Figure 21, (SEQ ID No. 54-88) shows possible degenerate oligonucleotide probes for this purpose, and Figure 23,
20 (SEQ ID Nos. 90-119), lists possible PCR primers. DNA sequence and polypeptide sequence should be obtainable by this means as with GGF-II, and also DNA constructs and expression vectors incorporating such DNA sequence, host cells genetically altered by incorporating such
25 constructs/vectors, and protein obtainable by cultivating such host cells. The invention envisages such subject matter.

I. Design and Synthesis of oligonucleotide Probes and
   Primers
30   Degenerate DNA oligomer probes were designed by backtranslating the amino acid sequences (derived from the peptides generated from purified GGF protein) into nucleotide sequences. Oligomers represented either the coding strand or the non-coding strand of the DNA sequence.

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When serine, arginine or leucine were included in the
oligomer design, then two separate syntheses were prepared
to avoid ambiguities. For example, serine was encoded by
either TCN or AGY as in 537 and 538 or 609 and 610.
5 Similar codon splitting was done for arginine or leucine
(e.g. 544, 545). DNA oligomers were synthesized on a
Biosearch 8750 4-column DNA synthesizer using ß cyanoethyl
chemistry operated at 0.2 micromole scale synthesis.
Oligomers were cleaved off the column (500 angstrom CpG
10 resins) and deprotected in concentrated ammonium hydroxide
for 6-24 hours at 55-60'C. Deprotected oligomers were
dried under vacuum (Speedvac) and purified by
electrophoresis in gels of 15% acrylamide (20 mono : 1
bis), 50 mM Tris-borate-EDTA buffer containing 7M urea.
15 Full length oligomers were detected in the gels by UV
shadowing, then the bands were excised and DNA oligomers
eluted into 1.5 μls H20 for 4-16 hours with shaking. The
eluate was dried, redissolved in 0.1 μl H_2O and absorbance
measurements were taken at 260nm.
20   Concentrations were determined according to the
following formula:

(A 260 x units/μl) (60.6/length) = x μM

All oligomers were adjusted to 50 μM concentration
by addition of H_2O.
25   Degenerate probes designed as above are shown in
Figure 21, (SEQ ID Nos. 54-88).

PCR primers were prepared by essentially the same
procedures that were used for probes with the following
modifications. Linkers of thirteen nucleotides containing
30 restriction sites were included at the 5' ends of the
degenerate oligomers for use in cloning into vectors. DNA
synthesis was performed at 1 micromole scale using 1,000
angstrom CpG resins and inosine was used at positions where
all four nucleotides were incorporated normally into
35 degenerate probes. Purifications of PCR primers included

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an ethanol precipitation following the gel electrophoresis
purification.

II. Library Construction and Screening

A bovine genomic DNA library was purchased from
5   Stratagene (Catalogue Number: 945701). The library
contained 2 x 10^6 15-20kb Sau3Al partial bovine DNA
fragments cloned into the vector lambda DashII. A bovine
total brain CDNA library was purchased from Clonetech
(Catalogue Number: BL 10139). Complementary DNA libraries
10 were constructed (In Vitrogen; Stratagene) from mRNA
prepared from bovine total brain, from bovine pituitary and
from bovine posterior pituitary. In Vitrogen prepared two
cDNA libraries: one library was in the vector lambda g10,
the other in vector pcDNAI (a plasmid library). The
15 Stratagene libraries were prepared in the vector lambda
unizap. Collectively, the cDNA libraries contained 14
million primary recombinant phage.

The bovine genomic library was plated on E. coli K12
host strain LE392 on 23 x 23 cm plates (Nunc) at 150,000 to
20 200,000 phage plaques per plate. Each plate represented
approximately one bovine genome equivalent. Following an
overnight incubation at 37°c, the plates were chilled and
replicate filters were prepared according to procedures of
Grunstein and Hogness ((1975) PNAS (USA) 72:3961). Four
25 plaque lifts were prepared from each plate onto uncharged
nylon membranes (Pall Biodyne A or MSI Nitropure). The DNA
was immobilized onto the membranes by cross-linking under
UV light for 5 minutes or, by baking at 80°C under vacuum
for two hours. DNA probes were labelled using T4
30 polynucleotide kinase (New England Biolabs) with gamma ^{32}P
ATP (New England Nuclear; 6500 Ci/mmol) according to the
specifications of the suppliers. Briefly, 50 pmols of
degenerate DNA oligomer were incubated in the presence of
600 μCi gamma ^{32}P-ATP and 5 units T4 polynucleotide kinase

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for 30 minutes at 37°C.   Reactions were terminated, gel
electrophoresis   loading   buffer   was   added   and   then
radiolabelled probes were purified by electrophoresis. ^{32}P
labelled probes were excised from gel slices and eluted
5   into water.   Alternatively, DNA probes were labelled via
PCR amplification by incorporation of α^{32}P-dATP or α^{32}P dCTP
according to the protocol of Schowalter and Sommer ((1989)
Anal. Biochem 177:90-94).   Probes labelled in PCR reactions
were purified by desalting on Sephadex G-150 columns.

10   Prehybridization and hybridization were performed in
GMC   buffer   (0.52M   NaPi,   7%   SDS,   1%   BSA,   1.5mM   EDTA,
0.1MNaCl 10μg/μl TRNA).   Washing was performed in buffer A
oligowash (160μl 1M Na2HPO$_4$, 200 μl 20% SDS, 8.0 μl 0.5m
EDTA, 100 μl 5M NaCl, 3632 μl H20).   Typically, 20 filters
15   (400 sq. centimetres each) representing replicate copies of
ten bovine genome equivalents were incubated in 200 μl
hybridization   solution   with   100   pmols   of   degenerate
oligonucleotide   probe   (128-512   fold   degenerate).
Hybridization was allowed to occur overnight at 5°C below
20   the   minimum   melting   temperature   calculated   for   the
degenerate probe.   The   calculation of   minimum   melting
temperature assumes 2°C for an AT pair and 4°C for a GC
pair.

        Filters were washed in repeated changes of oligowash
25   at the hybridization temperatures for four to five hours
and finally, in 3.2M tetramethylammonium chloride, 1% SDS
twice for 30 min at a temperature dependent on the DNA
probe length.   For 20mers, the final wash temperature was
60°C.   Filters were mounted, then exposed to X-ray film
30   (Kodak XAR5) using   intensifying   screens   (Dupont Cronex
Lightening Plus).   Usually, a   three   to   five   day   film
exposure at minus 80°C was sufficient to detect duplicate
signals in these library screens.   Following analysis of
the results, filters could be stripped and reprobed.
35 Filters were stripped by incubating through two successive

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cycles of fifteen minutes in a microwave oven at full power
in a solution of 1% SDS containing 10mM EDTA pH8. Filters
were taken through at least three to four cycles of
stripping and reprobing with various probes.

5   III. <u>Recombinant Phage Isolation, Growth and DNA</u>
          <u>Preparation</u>
          These procedures followed standard protocol as
    described in <u>Recombinant DNA</u> (Maniatis et al. Recombinant
    DNA <u>2</u>:60-62:81).

10 IV.   <u>Analysis of Isolated Clones Using DNA Digestion and</u>
          <u>Southern Blots</u>
          Recombinant Phage DNA samples (2 micrograms) were
    digested according to conditions recommended by the
    restriction endonuclease supplier (New England Biolabs).
15 Following a four hour incubation at 37°C, the reactions
    products were precipitated in the presence of 0.1M sodium
    acetate and three volumes of ethanol. Precipitated DNA was
    collected by centrifugation, rinsed in 75% ethanol and
    dried. All resuspended samples were loaded onto agarose
20 gels (typically 1% in TAE buffer; 0.04M Tris acetate, 0.
    002M EDTA). Gel runs were at 1 volt per centimetre from 4
    to 20 hours. Markers included lambda Hind III DNA
    fragments and/or ØX174HaeIII DNA fragments (New England
    Biolabs). The gels were stained with 0.5 micrograms/µl of
25 ethidium bromide and photographed. For southern blotting,
    DNA was first depurinated in the gel by treatment with
    0.125 N HCl, denatured in 0.5 N NaOH and transferred in 20x
    SSC (3M sodium chloride, 0.03 M sodium citrate) to
    uncharged nylon membranes. Blotting was done for 6 hours
30 up to 24 hours, then the filters were neutralized in 0.5M
    Tris HCl pH 7.5, 0.15 M sodium chloride, then rinsed
    briefly in 50 mM Tris-borate EDTA.
          For cross-linking, the filters were wrapped first in
    transparent plastic wrap, then the DNA side exposed for

five minutes to an ultraviolet light. Hybridization and
washing was performed as described for library screening
(see section 2 of this Example). For hybridization
analysis to determine whether similar genes exist in other
5 species slight modifications were made. The DNA filter was
purchased from Clonetech (Catalogue Number 7753-1) and
contains 5 micrograms of EcoRI digested DNA from various
species per lane. The probe was labelled by PCR
amplification reactions as described in section 2 above,
10 and hybridizations were done in 80% buffer B(2g
polyvinylpyrrolidine, 2g Ficoll-400, 2g bovine serum
albumin, 50μl 1M Tris-HCl(pH 7.5) 58g NaCl, 1g sodium
pyrophosphate, log sodium dodecyl sulfate, 950μl H_2O)
containing 10% dextran sulfate. The probes were denatured
15 by boiling for ten minutes then rapidly cooling in ice
water. The probe was added to the hybridization buffer at
10^6 dpm ^{32}P per μl and incubated overnight at 60°C. The
filters were washed at 60°C first in buffer B followed by
2X SSC, 0.1% SDS then in 1x SSC, 0.1% SDS. For high
20 stringency, experiments, final washes were done in 0.1 x
SSC, 1% SDS and the temperature raised to 65°C.

Southern blot data were used to prepare a
restriction map of the genomic clone and to indicate which
subfragments hybridized to the GGF probes (candidates for
25 subcloning).

V.   Subcloning of Pieces of DNA Homologous to Hybridization
     Probes

DNA digests (e.g. 5 micrograms) were loaded onto 1%
agarose gels then appropriate fragments excised from the
30 gels following staining. The DNA was purified by
adsorption onto glass beads followed by elution using the
protocol described by the supplier (Bio 101). Recovered
DNA fragments -(100-200 ng) were ligated into linearized
dephosphorylated vectors, e.g. pT3T7 (Ambion), which is a
35 derivative of pUC18, using T4 ligase (New England Biolabs).

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This vector carries the E. <u>coli</u> ß lactamase gene, hence, transformants can be selected on plates containing ampicillin. The vector also supplies ß-galactosidase complementation to the host cell, therefore
5 non-recombinants (blue) can be detected using isopropylthiogaloctoside and Bluogal (Bethesda Research Labs). A portion of the ligation reactions was used to transform <u>E</u>. <u>coli</u> K12 XL1 blue competent cells (Stratagene Catalogue Number: 200236) and then the transformants were
10 selected on LB plates containing 50 micrograms per μl ampicillin. White colonies were selected and plasmid mini preps were prepared for DNA digestion and for DNA sequence analysis. Selected clones were retested to determine if their insert DNA hybridized with the GGF probes.

15 VI. <u>DNA Sequencing</u>

Double stranded plasmid DNA templates were prepared from double stranded plasmids isolated from 5μl cultures according to standard protocols. Sequencing was by the dideoxy chain termination method using Sequenase 2.0 and a
20 dideoxynucleotide sequencing kit (US Biochemical) according to the manufacturers protocol (a modification of Sanger et al. (1977) PNAS (USA) <u>74</u>:5463). Alternatively, sequencing was done in a DNA thermal cycler (Perkin Elmer, model 4800) using a cycle sequencing kit (New England Biolabs; Bethesda
25 Research Laboratories) and was performed according to manufacturers instructions using a 5'-end labelled primer. Sequence primers were either those supplied with the sequencing kits or were synthesized according to sequence determined from the clones. Sequencing reactions were
30 loaded on and resolved on 0.4mm thick sequencing gels of 6% polyacylamide. Gels were dried and exposed to X-Ray film. Typically, 35S was incorporated when standard sequencing kits were used and a ^{32}P end labelled primer was used for cycle sequencing reactions. Sequences were read into a DNA

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sequence editor from the bottom of the gel to the top (5'
direction to 3') and data were analyzed using programs
supplied by Genetics Computer Group (GCG, University of
Wisconsin).

5   VII.   RNA Preparation and PCR Amplification

         Open reading frames detected in the genomic DNA and
which   contained   sequence   encoding   GGF   peptides   were
extended via PCR amplification of pituitary RNA.   RNA was
prepared from frozen bovine tissue (Pelfreeze) according to
10   the guanidine neutral-CsCl chloride procedure (Chirgwin et.
al. (1979) Biochemistry 18:5294).   Polyadenylated RNA was
selected by oligo-dT cellulose column chromatography (Aviv
and Leder. (1972) PNAS (USA) 69:1408).

         Specific target nucleotide sequences were amplified
15   beginning with either total RNA or polyadenylated RNA
samples that had been converted to cDNA using the Perkin
Elmer PCR/RNA Kit Number: N808-0017.   First strand reverse
transcription reactions used 1 μg template RNA and either
primers of oligo dT with restriction enzyme recognition
20   site   linkers   attached   or   specific   antisense   primers
determined from cloned sequences with restriction sites
attached.   To produce the second strand, the primers either
were   plus   strand   unique   sequences   as   used   in   3'   RACE
reactions (Frohman et. al. (1988) PNAS (USA) 85:8998) or
25   were oligo dT primers with restriction sites attached if
the   second   target   site   had   been   added   by   terminal
transferase tailing first strand reaction products with
dATP (e.g.   5'   race   reactions, Frohman et. al.,   ibid).
Alternatively,   as   in   anchored   PCR   reactions   the   second
30   strand   primers   were   degenerate,   hence,   representing
particular peptide sequences.

         The amplification profiles followed the following
general scheme: 1) five minutes soak file at 95°C; 2)
thermal cycle file of 1 minute, 95°C; 1 minute ramped down
35   to an annealing temperature of 45°C, 50°C or 55°C; maintain

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the annealing temperature for one minute; ramp up to 72°C over one minute; extend at 72°C for one minute or for one minute plus a 10 second auto extension; 3) extension cycle at 72°C, five minutes, and; 4) soak file 40°C for infinite
5   time. Thermal cycle files (#2) usually were run for 30 cycles. Sixteen μl of each 100 μl amplification reaction was analyzed by electrophoresis in 2% Nusieve 1% agarose gels run in TAE buffer at 4 volts per centimetre for three hours. The gels were stained, then blotted to uncharged
10 nylon membranes which were probed with labelled DNA probes that were internal to the primers.

        Specific sets of DNA amplification products could be identified in the blotting experiments and their positions used as a guide to purification and reamplification. When
15 appropriate, the remaining portions of selected samples were loaded onto preparative gels, then following electrophoresis four to five slices of 0.5 mm thickness (bracketing the expected position of the specific product) were taken from the gel. The agarose was crushed, then
20 soaked in 0.5 μl of electrophoresis buffer from 2-16 hours at 40°C. The crushed agarose was centrifuged for two minutes and the supernate was transferred to fresh tubes.

        Reamplification was done on five microlitres (roughly 1% of the product) of the eluted material using
25 the same sets of primers and the reaction profiles as in the original reactions. When the reamplification reactions were completed, samples were extracted with chloroform and transferred to fresh tubes. Concentrated restriction enzyme buffers and enzymes were added to the reactions in
30 order to cleave at the restriction sites present in the linkers. The digested PCR products were purified by gel electrophoresis, then subcloned into vectors as described in the subcloning section above. DNA sequencing was done described as above.

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VII. DNA Sequence Analysis

        DNA sequences were assembled using a fragment
assembly program and the amino acid sequences deduced by
the GCG programs GelAssemble, Map and Translate. The
5 deduced protein sequences were used as a query sequence to
search protein sequence databases using WordSearch.
Analysis was done on a VAX Station 3100 workstation
operating under VMS 5.1. The database search was done on
SwissProt release number 21 using GCG Version 7.0.

10 VII. Results
        As indicated, to identify the DNA sequence encoding
bovine GGF-II degenerate oligonucleotide probes were
designed from GGF-II peptide sequences. GGF-II 12 (SEQ ID
No. 44), a peptide generated via lysyl endopeptidase
15 digestion of a purified GGF-II preparation (see Figures 11
and 12) showed strong amino acid sequence homology with
GGF-I 07 (SEQ ID No. 39), a tryptic peptide generated from
a purified GGF-I preparation. GGF-II 12 was thus used to
create ten degenerate oligonucleotide probes (see oligos
20 609, 610 and 649 to 656 in Figure 21, SEQ ID Nos. 69-71 and
79, respectively). A duplicate set of filters were probed
with two sets (set 1=609, 610; set 2=649-656) of probes
encoding two overlapping portions of GGF-II 12.
Hybridization signals were observed, however, only one
25 clone hybridized to both probe sets. The clone (designated
GGF2BG1) was purified.
        Southern blot analysis of DNA from the phage clone
GGF2BG1 confirmed that both sets of probes hybridized with
that bovine DNA sequence, and showed further that both
30 probes reacted with the same set of DNA fragments within
the clone. Based on those experiments a 4 kb Eco RI
sub-fragment of the original clone was identified,
subcloned and partially sequenced. Figure 22 shows the
nucleotide sequence and the deduced amino acid sequence

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(SEQ ID No. 89) of the initial DNA sequence readings that
included the hybridization sites of probes 609 and 650, and
confirmed that a portion of this bovine genomic DNA encoded
peptide 12 (KASLADSGEYM).

5   Further sequence analysis demonstrated that GGF-II
12 resided on a 66 amino acid open reading frame (see
below) which has become the starting point for the
isolation of overlapping sequences representing a putative
bovine GGF-II gene and a cDNA.

10   Several PCR procedures were used to obtain
additional coding sequences for the putative bovine GGF-II
gene. Total RNA and oligo dT-selected (poly A containing)
RNA samples were prepared from bovine total pituitary,
anterior pituitary, posterior pituitary, and hypothalamus.

15 Using primers from the list shown in Figure 23 (SEQ ID No.
109-119) one-sided PCR reactions (RACE) were used to
amplify cDNA ends in both the 3' and 5' directions, and
anchored PCR reactions were performed with degenerate
oligonucleotide primers representing additional GGF-II

20 peptides. Figure 24 summarizes the contiguous DNA
structures and sequences obtained in those experiments.
From the 3' RACE reactions, three alternatively spliced
CDNA sequences were produced, which have been cloned and
sequenced. A 5' RACE reaction led to the discovery of an

25 additional exon containing coding sequence for at least 52
amino acids. Analysis of that deduced amino acid sequence
revealed peptides GGF-II-6 and a sequence similar to
GGF-I-18 (see below). The anchored PCR reactions led to
the identification of (cDNA) coding sequences of peptides

30 GGF-II-1, 2, 3 and 10 contained within an additional cDNA
segment of 300 bp. The 5' limit of this segment (i.e.
segment E, see Fig. 31) is defined by the oligonucleotide
which encodes peptide GGF-II-1 and which is used in the PCR
reaction. (Additional 5' sequence data exists as described

35 for the human clone in Example 6.) Thus this clone

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contains nucleotide sequences encoding six out of the existing total of nine novel GGF-II peptide sequences.

The cloned gene was characterized first by constructing a physical map of GGF2BG1 that allowed
5 positioning the coding sequences as they were found (see below, Figure 25). DNA probes from the coding sequences described above have been used to identify further DNA fragments containing the exons on this phage clone and to identify clones that overlap in both directions. The
10 putative bovine GGF-II gene is divided into at least 5 coding segments, but only coding segments A and B have been defined as exons and sequenced and mapped thus far. The summary of the contiguous coding sequences identified is shown in Figure 26. The exons are listed (alphabetically)
15 in the order of their discovery. It is apparent from the intron/exon boundaries that exon B may be included in cDNAs that connect coding segment E and coding segment A. That is, exon B cannot be spliced out without compromising the reading frame. Therefore, we suggest that three
20 alternative splicing patterns can produce putative bovine GGF-II cDNA sequences 1, 2 and 3. The coding sequences of these, designated GGF2BPP1.CDS, GGF2BPP2.CDS and GGF2BPP3.CDS, respectively, are given in Figures 28a (SEQ ID No. 133), 28b (SEQ ID No. 134) and 28c (SEQ ID No. 135),
25 respectively. The deduced amino acid sequence of the three cDNAs is also given in Figures 28a, 28b and 28c (SEQ ID Nos. 133-135, respectively).

The three deduced structures encode proteins of lengths 206, 281 and 257 amino acids. The first 183
30 residues of the deduced protein sequence are identical in all three gene products. At position 184 the clones differ significantly. A codon for glycine GGT in GGF2BPP1 also serves as a splice donor for GGF2BPP2 and GGF2BPP3, which alternatively add on exons C, C/D, C/D' and D or C, C/D and
35 D, respectively shown in Figure 33 (SEQ ID No. 149).

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GGF2BPP1 is a truncated gene product which is generated by reading past the coding segment a splice junction into the following intervening sequence (intron). This represents coding segment A' in Figure 31 (SEQ ID Nos. 140, 168). The

5 transcript ends adjacent to a canonical AATAAA polyadenylation sequence, and we suggest that this truncated gene product represents a bona fide mature transcript. The other two longer gene products share the same 3' untranslated sequence and polyadenylation site.

10   All three of these molecules contain six of the nine novel GGF-II peptide sequences (see Figure 12) and another peptide is highly homologous to GGF-I-18 (see Figure 27). This finding gives a high probability that this recombinant molecule encodes at least a portion of bovine GGF-II.

15 Furthermore, the calculated isoelectric points for the three peptides are consistent with the physical properties of GGF-I and II. Since the molecular size of GGF-2 is roughly 60 kd, the longest of the three cDNAs should encode a protein with nearly one-half of the predicted number of

20 amino acids.

          A probe encompassing the B and A exons was labelled via PCR amplification and used to screen a cDNA library made from RNA isolated from bovine posterior pituitary. One clone (GGF2BPP5) showed the pattern indicated in Figure

25 30 and contained an additional DNA coding segment (G) between coding segments A and C. The entire nucleic acid sequence is shown in Figure 32 (SEQ ID No. 148). The predicted translation product from the longest open reading frame is 241 amino acids. A portion of a second cDNA

30 (GGF2BPP4) was also isolated from the bovine posterior pituitary library using the probe described above. This clone showed the pattern indicated in Figure 30. This clone is incomplete at the 5' end, but is a splicing variant in the sense that it lacks coding segments G and D.

35 BPP4 also displays a novel 3' end with regions H, K and L

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beyond region C/D. The sequence of BPP4 is shown in Figure
34 (SEQ ID no. 150).

EXAMPLE 5

GGF Sequences in Various Species

5   Computer database searching has not revealed any
meaningful similarities between any predicted GGF
translation products and known protein sequences. This
suggests that GGF-II is the first member of a new family or
superfamily of proteins.   In high stringency cross
10 hybridization studies (DNA blotting experiments) with other
mammalian DNAs we have shown clearly that DNA probes from
this bovine recombinant molecule can readily detect
specific sequences in a variety of samples tested.   A
highly homologous sequence is also detected in human
15 genomic DNA. The autoradiogram is shown in Figure 29. The
signals in the lanes containing rat and human DNA represent
the rat and human equivalents of GGF, the sequences of
which have been recently reported by Holmes et al. ((1992)
Science 256:1205) and Wen et al. ((1992) Cell 69:559).

20   EXAMPLE 6

Isolation of a Human Sequence Encoding Human GGF2

          Several human clones containing sequences homologous
to the bovine GGFII coding segment E were isolated by
screening a human cDNA library prepared from brain stem
25 (Stratagene catalog #935206).   This strategy was pursued
based upon the strong link between most of the GGF2
peptides (unique to GGF2) and the predicted peptide
sequence from clones containing the bovine E segment. This
library was screened as described in Example 4, Section II
30 using the oligonucleotide probes 914-919 listed below.
914   TCGGGCTCCATGAAGAAGATGTA
915   TCCATGAAGAAGATGTACCTGCT
916   ATGTACCTGCTGTCCTCCTTGA

917   TTGAAGAAGGACTCGCTGCTCA
918   AAAGCCGGGGGCTTGAAGAA
919   ATGARGTGTGGGCGGCGAAA

Clones detected with these probes were further
analyzed by hybridization. A probe derived from coding
segment A (see Figure 21), which was produced by labeling
a polymerase chain reaction (PCR) product from segment A,
was also used to screen the primary library. Several
clones that hybridized with both A and E derived probes
were selected and one particular clone, GGF2HBS5, was
selected for further analysis. This clone is represented
by the pattern of coding segments (EBACC/D'D as shown in
Figure 31). The E segment in this clone is the human
equivalent of the truncated bovine version of E shown in
Figure 37. GGF2HBS5 is the most likely candidate to encode
GGFII of all the "putative" GGFII candidates described.
The length of coding sequence segment E is 786 nucleotides
plus 264 bases of untranslated sequence. The predicted
size of the protein encodied by GGF2HBS5 is approximately
423 amino acids (approximately 45 kilodaltons), which is
similar to the size of the deglycosylated form of GGF (see
Example 15). Additionally, seven of the GGFII peptides
listed in Figure 27 have equivalent sequences which fall
within the protein sequence predicted from region E.
Peptides II-6 and II-12 are exceptions, which fall in
coding segment B and coding segment A, respectively. RNA
encoding the GGF2HBS5 protein was produced in an _in vitro_
transcription system driven by the bacteriophage T7
promoter resident in the vector (Bluescript SK [Stratagene
Inc.] see Figure 44) containing the GGF2HBS5 insert. This
RNA can be translated in a cell free (rabbit reticulocyte)
translation system and the size of the protein product was
45 Kd. Additionally, the cell-free product has been
assayed in a Schwann cell mitogenic assay to confirm
biological activity. Schwann cells treated with

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conditioned medium show both increased proliferation as measured by incorporation of [125]-Uridine and phosphorylation on tyrosine of a protein in the 185 kilodalton range.

Thus the size of the product encoded by GGF2HBS5 and 5   the presence of DNA sequences which encode human peptides highly homologous to the bovine peptides shown in Figure 12 confirm that GGF2HBS5 encodes the human equivalent of bovine GGF2. The fact that conditioned media prepared from cells transformed with this clone elicits Schwann cell 10 mitogenic activity confirms that the GGFIIHBS5 gene product (unlike the BPP5 gene product) is secreted. Additionally the GGFBPP5 gene product seems to mediate the Schwann cell proliferation response via a receptor tyrosine kinase such as p185[erbB2] or a closely related receptor (see Example 13).

15   EXAMPLE 7
        Isolation of Human Sequences Related to Bovine GGF
        The result in Example 5 indicates that GGF related sequences from human sources can also be easily isolated by using DNA probes derived from bovine GGF sequences.
20 Alternatively, the procedure described by Holmes et al. ((1992) Science 256:1205) can be used. In this example a human protein (heregulin α) which binds to and activates the p185[erbB2] receptor (and is related to GGF) is purified from a tumor cell line and the derived peptide sequence is 25 used to produce oligonucleotide probes which were utilized to clone the cDNAs encoding heregulin. This is a similar approach to that used in examples 1-4 for the cloning of GGF sequences from pituitary cDNAs. The heregulin protein and complementary DNAs were isolated according to the 30 following procedures. Heregulin was purified from medium conditioned by MDA-MB-231 breast cancer cells (ATCC #HTB 26) grown on Percell Biolytica microcarrier beads (Hyclone Labs). The medium (10 liters) was concentrated ~25-fold by filtration through a membrane (10-kD cutoff) (Millipore)

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and clarified by centrifugation and filtration through a filter (0.22 μm). The filtrate was applied to a heparin Sepharose column (Pharmacia) and the proteins were eluted with steps of 0.3, 0.6, and 0.9 M NaCl in
5   phosphate-buffered saline. Activity in the various chromatographic fractions was measured by quantifying the increase in tyrosine phosphorylation of p185^{erbB2} in MCF-7 breast tumor cells (ATCC # HTB 22). MCF-7 cells were plated in 24-well Costar plates in F12 (50%) Dulbecco's
10 minimum essential medium (50%) containing serum (10%) (10^5 cells per well), and allowed to attach for at least 24 hours. Prior to assay, cells were transferred into medium without serum for a minimum of 1 hour. Column fractions (10 to 100 μl) were incubated for 30 min. at 37°.
15 Supernatants were then aspirated and the reaction was stopped by the addition of SDS-PAGE sample buffer 100 μl). Samples were heated for 5 min. at 100°C, and portions (10 to 15 μl) were applied to a tris-glycine gel (4 to 20%) (Novex). After electrophoresis, proteins were
20 electroblotted onto a polyvinylidenedifluoride (PVDF) membrane and then blocked with bovine serum albumin (5%) in tris-buffered saline containing Tween-20 (0.05%) (TBST). Blots were probed with a monoclonal antibody (1:1000 dilution) to phosphotyrosine (Upstate Biotechnology) for a
25 minimum of 1 hour at room temperature. Blots were washed with TBST, probed with an antibody to mouse immunoglobulin G conjugated to alkaline phosphatase (Promega) (diluted 1:7500) for a minimum of 30 min. at room temperature. Reactive bands were visualized with
30 5-bromo-4-chloro-3-indoyl-1-phosphate and nitro-blue tetrazolium. Immunoblots were scanned with a Scan Jet Plus (Hewlett-Packard) densitometer. Signal intensities for unstimulated MCF-7 cells were 20 to 30 units. Fully stimulated p185^{erbB2} yielded signals of 180 to 200 units.
35 The 0.6 M NaCl pool, which contained most of the activity,

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was applied to a polyaspartic acid (PolyLC) column
equilibrated in 17 mM sodium phosphate (pH 6.8) containing
ethanol (30%). A linear gradient from 0.3 M to 0.6 M NaCl
in the equilibration buffer was used to elute bound
5   proteins. A peak of activity (at ~0.45 M NaCl) was further
fractionated on a C4 reversed-phase column (SynChropak
RP-4) equilibrated in buffer containing TFA (0.1%) and
acetonitrile (15%). Proteins were eluted from this column
with an acetonitrile gradient from 25 to 40% over 60 min.
10   Fractions (1 μl) were collected, assayed for activity, and
analyzed by SDS-PAGE on tris-glycine gels (4-20%, Novex).

     HPLC-purified HRG-α was digested with lysine C in
SDS (0.1%), 10 mM dithiothreitol, 0.1 M NH_4HCO_3 (pH 8.0) for
20 hours at 37°C and the resultant fragments were resolved
15   on a Synchrom C4 column (4000Å, 0.2 by 10 cm). The column
was equilibrated in 0.1% TFA and eluted with a 1-propanol
gradient in 0.1% TFA (Henzel et al. (1989) J. Biol. Chem.
264:15905). Peaks from the chromatographic run were dried
under vacuum and sequenced. One of the peptides (eluting
20   at   ~24%   1-propanol)   gave   the   sequence
[A]AEKEKTF[C]VNGGEXFMVKDLXNP (SEQ ID Nos 162). Residues in
brackets were uncertain and an X represents a cycle in
which it was not possible to identify the amino acid. The
initial yield was 8.5 pmol and the sequence did not
25   correspond to any known protein. Residues 1, 9, 15, and 22
were later identified in the cDNA sequence as cysteine.
Direct sequencing of the ~45-kD band from a gel that had
been overloaded and blotted onto a PVDF membrane revealed
a low abundance sequence XEXKE[G][R]GK[G]K[G]KKKEXGXG[K]
30   (SEQ ID No. 169) with a very low initial yield (0.2 pmol).
This corresponded to amino acid residues 2 to 22 of
heregulin-α (Fig. 31), suggesting that serine 2 is the
NH_2-terminus of proHRG-α. Although the NH_2 terminus was
blocked, it was observed that occasionally a small amount
35   of   a   normally   blocked   protein   may   not   be

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post-translationally modified. The NH$_2$ terminal assignment
was confirmed by mass spectrometry of the protein after
digestion with cyanogen bromide. The COOH-terminus of the
isolated protein has not been definitely identified;
5   however, by mixture sequencing of proteolytic digests, the
mature sequence does not appear to extend past residue 241.
Abbreviations for amino residues are: A, Ala; C, Cys; D,
Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L,
Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T.
10 Thr; V, Val; W, Trp; and Y, Tyr.

As a source of cDNA clones, an oligo(dT)-primed
λgt10 (Hurn et al. (1984) λgt10 and λgt11 DNA Cloning
Techniques: A Practical Approach) cDNA library was
constructed (Gubler and Hoffman. (1983) Gene 25:263) with
15 mRNA purified (Chirwin et al. (1979) Biochemistry 18:5294)
from MDA-MB-231 cells. The following eightfold degenerate
antisense deoxyoligonucleotide encoding the 13-amino acid
sequence AEKEKTFCVNGGE (SEQ ID No. 164) was designed on the
basis of human codon frequency optima (Lathe. (1985) J.
20 Mol. Biol. 183:1) and chemically synthesized:

5'-CTCGCC (G OR T) CC (A OR G) TTCAC (A OR G)
CAGAAGGTCTTCTCCTTCTCAGC-3' (SEQ ID No. 165). For the
purpose of probe design a cysteine was assigned to an
unknown residue in the amino acid sequence. The probe was
25 labeled by phosphorylation and hybridized under
low-stringency conditions to the cDNA library. The proHRG-
α protein was identified in this library. HRB-ß1 cDNA was
identified by probing a second oligo(dT)-primed λgt10
library made from MDA-MB-231 cell mRNA with sequences
30 derived from both the 5' and 3' ends of proHRG. Clone 13
IFig. 2A) was a product of screening a primed
(5'-CCTCGCTCCTTCTTCTTGCCCTTC-3' primer; proHRG-α antisense
nucleotides 33 to 56) MDA-MB-231 λgt10 library with 5' HRG
sequence. A sequence corresponding to the 5' end of clone
35 13 as the probe was used to identify proHRGß2 and proHRGß3

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in a third oligo(dT)-primed λgt10 library derived from MDA-MB-231 cell mRNA. Two cDNA clones encoding each of the four HRGs were sequenced (Sanger et al. (1977) PNAS (USA) 74:5463). Another cDNA designated clone 84 has an amino
5 acid sequence identical to proHRGβ2 through amino acid 420. A stop codon at position 421 is followed by a different 3'-untranslated sequence.

EXAMPLE 8
Isolation of a Further Splicing Variant
10   The methods in Example 7 produced four closely related sequences (heregulin α, β1, β2, β3) which arise as a result of splicing variation. Peles et al. ((1992) Cell 69:205) and Wen et al. ((1992) Cell 69:559) have isolated another splicing variant (from rat) using a similar
15 purification and cloning approach to that described in Examples 1-4 and 7 involving a protein which binds to p185^{erbB2}. The cDNA clone was obtained as follows (via the purification and sequencing of a p185^{erbB2} binding protein from a transformed rat fibroblast cell line).
20   A p185^{erbB2} binding protein was purified from conditioned medium as follows. Pooled conditioned medium from three harvests of 500 roller bottles (120 liters total) was cleared by filtration through 0.2 μ filters and concentrated 31-fold with a Pelicon ultrafiltration system
25 using membranes with a 20kd molecular size cutoff. All the purification steps were performed by using a Pharmacia fast protein liquid chromatography system. The concentrated material was directly loaded on a column of heparin-Sepharose (150 μl, preequilibrated with
30 phosphate-buffered saline (PBS)). The column was washed with PBS containing 0.2 M NaCl until no absorbance at 280 nm wavelength could be detected. Bound proteins were then eluted with a continuous gradient (250 μl) of NaCl (from 0.2 M to 1.0 M), and 5 μl fractions were collected.

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Samples (0.01 μl of the collected fractions) were used for the quantitative assay of the kinase stimulatory activity. Active fractions from three column runs (total volume = 360 μl) were pooled, concentrated to 25 μl by using a YM10

5   ultrafiltration membrane (Amicon, Danvers, MA), and ammonium sulfate was added to reach a concentration of 1.7 M.   After clearance by centrifugation (10,000 x g, 15 min.), the pooled material was loaded on a phenyl-Superose column (HR10/10, Pharmacia). The column was developed with

10 a 45 μl gradient of $(NH_4)_2SO_4$ (from 1.7 M to no salt) in 0.1 M Na_2PO_4 (pH 7.4), and 2 μl fractions were collected and assayed (0.002 μl per sample) for kinase stimulation (as described in Example 7). The major peak of activity was pooled and dialyzed against 50 mM sodium phosphate buffer

15 (pH 7.3).   A Mono-S cation-exchange column (HR5/5, Pharmacia) was preequilibrated with 50 mM sodium phosphate. After loading the active material (0.884 mg of protein; 35 μl), the column was washed with the starting buffer and then developed at a rate of 1 μl/min. with a gradient of

20 NaCl.   The kinase stimulatory activity was recovered at 0.45-0.55 M salt and was spread over four fractions of 2 μl each.   These were pooled and loaded directly on a Cu^{+2} chelating columns (1.6 μl, HR2/5 chelating Superose, Pharmacia). Most of the proteins adsorbed to the resin,

25 but they gradually eluted with a 30 μl linear gradient of ammonium chloride (0-1 M). The activity eluted in a single peak of protein at the range of 0.05 to 0.2 M NH_4Cl. Samples from various steps of purification were analyzed by gel electrophoresis followed by silver staining using a kit

30 from ICN (Costa Mesa, CA), and their protein contents were determined with a Coomassie blue dye binding assay using a kit from Bio-Rad (Richmond, CA).

The p44 protein (10 μg) was reconstituted in 200 μl of 0.1 M ammonium bicarbonate buffer (pH 7.8). Digestion

35 was   conducted   with   L-1-tosyl-amide   2-phenylethyl

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chloromethyl ketone-treated trypsin (Serva) at 37°C for 18 hr. at an enzyme-to-substrate ratio of 1:10. The resulting peptide mixture was separated by reverse phase HPLC and monitored at 215 nm using a Vydac C4 micro column (2.1 mm
5 i.d. x 15 cm, 300) and an HP 1090 liquid chromatographic system equipped with a diode-array detector and a workstation. The column was equilibrated with 0.1% trifluoroacetic acid (mobile phase A), and elution was effected with a linear gradient from 0%-55% mobile phase B
10 (90% acetonitrile in 0.1% trifluoroacetic acid) over 70 min. The flow rate was 0.2 μl/min. and the column temperature was controlled at 25°C. One-third aliquots of the peptide peaks collected manually from the HPLC system were characterized by N-terminal sequence analysis by Edman
15 degradation. The fraction eluted after 27.7 min. (T27.7) contained mixed amino acid sequences and was further rechromatographed after reduction as follows: A 70% aliquot of the peptide fraction was dried in vacuo and reconstituted in 100 μl of 0.2 M ammonium bicarbonate
20 buffer (pH 7.8). DTT (final concentration 2 mM) was added to the solution, which was then incubated at 37°C for 30 min. The reduced peptide mixture was then separated by reverse-phase HPLC using a Vydac column (2.1 mm i.d. x 15 cm). Elution conditions and flow rat'were identical to
25 those described above. Amino acid sequence analysis of the peptide was performed with a Model 477 protein sequencer (Applied Biosystems, Inc., Foster City, CA) equipped with an on-line phenylthiohydantoin (PTH) amino acid analyzer and a Model 900 data analysis system (Hunkapiller et al.
30 (1986)). The protein was loaded onto a trifluoroacetic acid-treated glass fiber disc precycled with polybrene and NaCl. The PTH-amino acid analysis was performed with a micro liquid chromatography system (Model 120) using dual syringe pumps and reverse-phase (C-18) narrow bore columns
35 (Applied Biosystems, 2.1 mm x 250 mm).

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RNA was isolated from Rat1-EJ cells by standard procedures (Maniatis et al. (1982) Molecular Cloning: A Laboratory Manual) and poly (A)$^+$ was selected using an mRNA Separator kit (Clontech Lab, Inc., Palo, Alto, CA). cDNA
5 was synthesized with the Superscript kit (from BRL Life Technologies, Inc., Bethesda, MS). Column-fractionated double-strand cDNA was ligated into an Sall- and NacI-digested pJT-2 plasmid vector, a derivative of the pCD-X vector (Okayama and Berg (1983) Mol. Cell Biol.
10 $\underline{3}$:280) and transformed into DH10B $\underline{E}$. $\underline{coli}$ cells by electroporation (Dower et al. (1988) Nucl. Acids Res. $\underline{16}$:6127). Approximately 5 x 10^5 primary transformants were screened with two oligonucleotide probes that were derived from the protein sequences of the N-terminus of NDF
15 (residues 5-24) and the T40.4 tryptic peptide (residues 7-12). Their respective sequences were as follows (N indicates all 4 nt):

(1)   5'-ATA GGG AAG GGC GGG GGA AGG GTC NCC CTC NGC
                                                A   T
20   AGG GCC GGG CTT GCC TCT GGA GCC TCT-3'

(2)   5'-TTT ACA CAT ATA TTC NCC-3'
         C   G   G   C
(1: SEQ ID No. 167; 2: SEQ ID No. 168)

The synthetic oligonucleotides were end-labeled with
25 [γ-^{32}P]ATP with T4 polynucleotide kinase and used to screen replicate sets of nitrocellulose filters. The hybridization solution contained 6 x SSC, 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 2 x Denhardt's solution, 50 μg/ml salmon sperm DNA, and 20%
30 formamide (for probe 1) or no formamide (for probe 2). The filters were washed at either 50°C with 0.5 x SSC, 0.2% SDS, 2 mM EDTA (for probe 1) or at 37°C with 2 x SSC, 0.2% SDS, 2 mM EDTA (for probe 2). Autoradiography of the filters gave ten clones that hybridized with both probes.

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These clones were purified by replating and probe
hybridization as described above.

The cDNA clones were sequenced using an Applied
Biosystems 373A automated DNA sequencer and Applied
5 Biosystems Taq DyeDeoxy™ Terminator cycle sequencing kits
following the manufacture's instructions.   In some
instances, sequences were obtained using [^{35}S]dATP
(Amersham) and Sequenase™ kits from U.S. Biochemicals
following the manufacturer's instructions. Both strands of
10 the cDNA clone 44 were sequenced by using synthetic
oligonucleotides as primers.   The sequence of the most 5'
350 nt was determined in seven independent cDNA clones.
The resultant clone demonstrated the pattern shown in
Figure 30 (NDF).

15   EXAMPLE 9
                      Other Possible Splicing Variants
                Alignment of the deduced amino acid sequences of the
cDNA clones and PCR products of the bovine, and the
published human (Fig. 31) and rat sequences show a high
20 level of similarity, indicating that these sequences are
derived from homologous genes within the three species.
The variable number of messenger RNA transcripts detectable
at the cDNA/PCR product level is probably due to extensive
tissue-specific splicing. The patterns obtained and shown
25 in Figure 30 suggests that other splicing variants exist.
A list of probable splicing variants is indicated as
followed. Many of these variants can be obtained by coding
segment specific probing of cDNA libraries derived from
different tissues.   Alternatively, the variants can be
30 assembled from specific (excised from) cDNA clones, PCR
products or genomic DNA regions via cutting and splicing
techniques known to one skilled in the art. These variant
sequences can be expressed in recombinant systems and the
recombinant products can be assayed to determine their

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level of Schwann cell mitogenic activity as well as their
ability to bind and activate the p185^{erbB2} receptor.

EXAMPLE 10

Functional elements of GGF

5   The deduced structures of family of GGF sequences
indicate that the longest forms (as represented by
GGF2BPP4) encode transmembrane proteins where the
extracellular part contains a domain which resembles
epidermal growth factor (see Carpenter and Wahl in Peptide
10 Growth Factors and Their Receptors I pp. 69-133, Springer-
Verlag, NY 1991). The positions of the cysteine residues
in coding segments C and C/D or C/D' peptide sequence are
conserved with respect to the analogous residues in the
epidermal growth factor (EGF) peptide sequence. This
15 suggests that the extracellular domain functions as a
receptor recognition and biological activation sites.
Several of the variant forms lack the H, K, and L coding
segments and thus may be expressed as secreted, diffusible
biologically active proteins. Likely structures are shown
20 in Figure 35.

Membrane bound versions of this protein may induce
Schwann cell proliferation if expressed on the surface of
neurons during embryogenesis or during nerve regeneration
(where the surfaces of neurons are intimately associated
25 with the surfaces of proliferating Schwann cells).

Secreted (non membrane bound) GGF's may act as
classically diffusible factors which can interact with
Schwann cells at some distance from their point of
secretion. An example of a secreted GGF is the protein
30 encoded by GGF2HBS5 (see example 6)

Other GGFs such as that encoded by GGF2BPP5 seem to
be non-secreted (see example 6). These GGFs may be injury
response forms which are released as a consequence of
tissue damage.

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EXAMPLE 11

Splicing variants with antiproliferative action

One particular splicing variant (GGF2BPP1) is described in Example 4. GGF2BPP1 is a truncated gene
5 product which is generated by reading past the coding segment A splice junction into the adjoining genomic sequence. This represents coding segment A' in Figure 31. The transcript ends near to a canonical AATAAA polyadenylation sequence. This splicing variant contains
10 regions F, E, B and A'. Other possible variants of this may lack region E (F, B, A'). As described in Example 10 regions C, C/D, or C/D' are homologous to EGF and are most likely to be the sites which are responsible for biological activity. GGF2BPP1 could retain receptor binding activity
15 yet lack the ability to activate the receptor. Such a ligand would function as an antagonist since it would compete with active GGF/p185 erbB2 ligands (eg. GGF2BPP5) for receptor binding. Other splicing variants such as those containing region E may also function as antagonists
20 as described above. The presence of an extra domain such as that which is encoded by region E may result in structural differences which would interfere with biological activity following receptor binding. GGF2BPP2 may also be an inhibitor molecule. The presence of region
25 C/D' in addition to region C/D in GGF2BPP2 adds sequence to the EGF related region which could potentially result in a protein which lacks biological activity. GGF2HBS11 is another potential inhibitor molecule. This clone was isolated from a human brainstem library using the same
30 methods and probes as described in Example 6 for the isolation of GGF2HBS5. The GGF2HBS11 clone contains a portion of region E which is flanked by new sequence which is not contained in any other known region. The lack of region C, C/D or C/D' suggests that GGF2HBS11 would also
35 lack biological activity.

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EXAMPLE 12
Purification of antiproliferative factors
from Recombinant Cells

In order to obtain antiproliferative factors to
5 assay for biological activity, the proteins can be
overproduced using cloned DNA. Several approaches can be
used. A recombinant E. coli cell containing the sequences
described in example 11 can be constructed. Expression
systems such as pNH8a (Stratagene, Inc.) can be used for
10 this purpose by following manufacturers procedures.
Alternatively, these sequences can be inserted in a
mammalian expression vector and an overproducing cell line
can be constructed. As an example, for this purpose DNA
encoding GGF2BPP1 can be expressed in COS cells or can be
15 expressed in Chinese hamster ovary cells using the pMSXND
expression vector (Lee and Nathans, J. Biol. Chem. 263,
3521-3527, (1981)). This vector containing GGF DNA
sequences can be transfected into host cells using
established procedures.

20   Transient expression can be examined or G418-
resistant clones can be grown in the presence of
methotrexate to select for cells that amplify the DHFR gene
(contained on the pMSXND vector) and, in the process, co-
amplify the adjacent protein encoding sequence. Because
25 CHO cells can be maintained in a totally protein-free
medium (Hamilton and Ham, in Vitro 13, 537-547 (1977)), the
desired protein can be purified from the medium. Western
analysis using the antisera produced in Example 9 can be
used to detect the presence of the desired protein in the
30 conditioned medium of the overproducing cells.

The desired protein can be purified from the E. coli
lysate or the CHO cell conditioned medium using the types
of procedures described in Example 1. The protein may be
assayed at various points in the procedure using a Western
35 blot assay.

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EXAMPLE 13

Design and assay of antiproliferative factors

As indicated above and in Figs. 35 and 39-45, the
GGF coding segments include regions with EGF-like homology.
5 These EGF-like domains can be required for the activation
of mitogenesis in the binding reaction between GGF ligands
containing such domains and the erbB2 receptor.
Comparisons of naturally occurring products of the GGF
coding sequences which confer mitogenic activity versus
10 those which confer antiproliferative activity, as disclosed
above, provide additional support for this. Consequently,
preferred antiproliferative factors are those which lack
these EGF-like domains. Antiproliferative factors designed
in this manner will lack all or part of the C, C/D, or C/D'
15 coding segments. Examples of such factors likely to have
antiproliferative activity using this design strategy are
shown in Fig. 37 and described in the summary of the
invention.

The recombinant proteins produced in Example 12
20 using the criterion described above may be assayed as
described hereafter. The Schwann cell mitogenic assay
described herein may be used to assay the expressed product
of the full length clone or any biologically active
portions thereof. Any member of the family of splicing
25 variant complementary DNA's derived from the GGF gene
(including the Heregulins) can be expressed in this manner
and assayed in the Schwann cell proliferation assay by one
skilled in the art. Antiproliferative activity in the GGF
assay can be examined by a competition assay (Chan et al.,
30 Science 254:1383 (1991)). Varying concentrations of
recombinant antiproliferative GGF variants (such as
GGF2BPP1) can be added to Schwann cell cultures in the
presence of GGF. The extent of antiproliferative activity
can be measured by comparing mitogenic activity of the
35 cultures to controls treated only with GGF. This will

provide a measure of dose dependent inhibition. The specificity of the response can be measured by examining the effect of varying concentrations of antiproliferative factor on the mitogenic activity of other growth factors

5 and their target cells (e.g. EGF). Antiproliferative activity of recombinant GGF variants can also be examined in breast tumour cells. Cell lines such as SK-BR-3 which proliferate in response to GGF's/p185^{erbB2} ligands can be assayed in a similar manner to that described above for

10 Schwann cells.

Crosslinking studies can be performed to determine whether I^{125} labelled GGF variants, which show antiproliferative activity (as described above), bind to the erbB2 receptor (Chan et al., Science 254:1383 (1991)).

15 Binding can be demonstrated by immunoprecipitation of the cross-linked protein with an antibody to the erbB2 receptor.

What is claimed is:

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Claims

1.   A   DNA   sequence   encoding   a   polypeptide
synthesized by the plasmid pGGF2HBS11 deposited with the
A.T.C.C. November 6, 1992 (A.T.C.C. Deposit No. 75347).

5   2.   A   polypeptide   encoded   by   pGGF2HBS11
deposited with the A.T.C.C. November 6, 1992 (A.T.C.C.
Deposit No. 75347).

3.   A polypeptide comprising the peptide encoded
by the E sequence (SEQ ID Nos. 137 and 163) and at least a
10 portion of the peptide encoded by the brain-derived DNA
sequences flanking the E encoding sequence on clone
pGGF2HBS11, ATCC Deposit No. 75347.

4.   The polypeptide of claim 3, wherein said
peptide encoded by the E sequence (SEQ ID Nos. 137 and 163)
15 has a deletion of the 48 N-terminal amino acids and wherein
said E-flanking peptide sequences contain between 20 and
100 N-terminal amino acids and between 30 and 50 C-terminal
amino acids encoded by said clone.

5.   The polypeptide of claim 3, wherein said
20 peptide encoded by the E sequence (SEQ ID Nos. 137 and 163)
has a deletion of the 48 N-terminal amino acids and wherein
said E-flanking peptide sequences contain between 25 and 70
N-terminal amino acids and between 30 and 45 C-terminal
amino acids encoded by said clone.

25   6.   A method for inhibiting proliferation of a
cell, said method comprising contacting said cell with a
polypeptide defined by the formula

VYBAZWX

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wherein VYBAZWX is composed of the polypeptide
segments shown in Figure 31 (SEQ ID Nos. 136-139, 141-147,
160, 161, and 163); wherein V comprises F, or is absent;
wherein Y comprises polypeptide segment E, or is absent;
5 wherein Z comprises polypeptide segment G or is absent;
wherein W comprises C or is absent; and wherein X comprises
polypeptide segments C/D HKL, C/D H, C/D HL, C/D D, C/D'
HL, C/D' HKL, C/D' H, C/D' D, C/D C/D' HKL, C/D C/D' H, C/D
C/D' HL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D'
10 D' H, C/D' D' HL, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D'
HL, C/D C/D' D' HKL, H, HL, or HKL.

7.   A method for inhibiting proliferation of a
cell, said method comprising contacting said cell with a
polypeptide comprising FBA polypeptide segments, FBA'
15 polypeptide segments, EBA polypeptide segments, EBA'
polypeptide segments, FEBA polypeptide segments, or FEBA'
polypeptide segments having the amino acid sequences
corresponding to polypeptide segments shown in Figure 31
(SEQ ID Nos. 136-140, 163, 168).

20   8.   A method of inhibiting proliferation of a
cell, said method comprising contacting said cell with a
polypeptide of claims 1-5.

9.   A method of inhibiting proliferation of a
cell, said method comprising contacting said cell with a
25 compound which specifically binds the p185^{erbB2} receptor of
said cell.

10.   The method of any one of claims 6-9, wherein
said cell is a cell of the nervous system.

11.   The method of claim 10, wherein said cell is
30 a glial cell.

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12.   The method of claim 11, wherein said cell is a Schwann cell.

13.   A method of any one of claims 6-9 wherein said cell is a cancer cell.

5   14.   A method of claim 13, wherein said cell is an adenocarcinoma cell.

15.   The method of any one of claims 6-9, wherein said method is used in the treatment or prophylaxis of a nervous disease or disorder.

10   16.   The method of any one of claims 6-9, wherein said cell is a cell in a mammal and said contacting is carried out by administering said peptide to said mammal for the prophylaxis or treatment of a pathophysiological condition in said mammal in which said condition involves
15 said cell.

17.   The method of any one of claims 6-9, wherein said method is used for the treatment or prophylaxis of a demyelination disease or disorder.

18.   A method as claimed in claim 16, wherein
20 said condition involves a disease of cell proliferation, such as a tumor or peripheral nerve damage caused by a cell tumor.

19.   The method of any one of claims 6-9, wherein said cell is in a mammal and said contacting is carried out
25 by administering said peptide to said mammal for the prophylaxis or treatment of a condition which involves a tumor of said cell, for example, neurofibromatosis, malignant Schwannomas or neurofibrosarcomas.

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20. The method of any one of claims 6-9, wherein said cell is in a mammal and said contacting is carried out by administering said peptide to said mammal for prophylaxis or treatment of a condition which involves a
5   meningioma, a bilateral acoustic neuroma, a astrocytoma, a retinoblastoma, a neuroglioma, a neuroblastoma, or a glioma.

21. A method for producing an antibody specific for a polypeptide, said method comprising
10   i) immunizing a mammal with a polypeptide selected from the group consisting of a polypeptide defined by the formula
                              VYBAZWX
        wherein VYBAZWX is composed of the polypeptide
15 segments shown in Figure 31 (SEQ ID Nos. 136-139, 141, 146, 147, 160, 161, and 163); wherein V comprises F, or is absent; wherein Y comprises polypeptide segment E, or is absent; wherein Z comprises polypeptide segment G or is absent; wherein W comprises C or is absent; and wherein X
20 comprises polypeptide segments H, HK, or HKL, and
            ii) purifying said antibody from tissue of said animal, or from a hybridoma made using said tissue.

22. A method for producing an antibody specific for a polypeptide, said method comprising
25   i) immunizing a mammal with a polypeptide selected from the group consisting of polypeptides defined by the formulas FBA, FBA', EBA, FEBA, FEBA', or EBA', wherein F, E, B, A and A' segments are defined by the amino acid sequences shown in Figure 31 (SEQ ID Nos. 136-140,
30 163, 168) and
            ii) purifying said antibody from tissue of said animal, or from a hybridoma made using said tissue.

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23. A method for detecting, in a sample, the presence of a molecule capable of binding to a receptor which binds to a polypeptide selected from the group consisting of polypeptides defined by the formula

5   VYBAZWX

wherein VYBAZWX is composed of the polypeptide segments shown in Figure 31 (SEQ ID Nos. 136-139, 141, 146, 147, 160, 161, and 163); wherein V comprises F, or is absent; wherein Y comprises polypeptide segment E, or is

10 absent; wherein Z comprises polypeptide segment G or is absent; wherein W comprises C or is absent; and wherein X comprises polypeptide segments H, HK, or HKL,

said method comprising the steps of

i) contacting said sample with said polypeptide

15 together with said receptor, and

ii) detecting competitive inhibition of the binding of said polypeptide to said receptor as an indication of the presence of a receptor binding molecule in said sample.

20   24. A method for detecting, in a sample, the presence of a molecule capable of binding to a receptor which binds to a polypeptide selected from the group consisting of polypeptides defined by the formulas FBA, FBA', EBA, FEBA, FEBA', or EBA', wherein F, E, B, A and A'

25 segments are defined by the amino acid sequences shown in Figure 31 (SEQ ID Nos. 136-140, 163, 168), said method comprising the steps of

i) contacting said sample with said polypeptide together with said receptor, and

30   ii) detecting competitive inhibition of the binding of said polypeptide to said receptor as an indication of the presence of a receptor binding molecule in said sample.

FIGURE 1

Hydroxylapatite HPLC

COUNTS I-125

Fraction No.
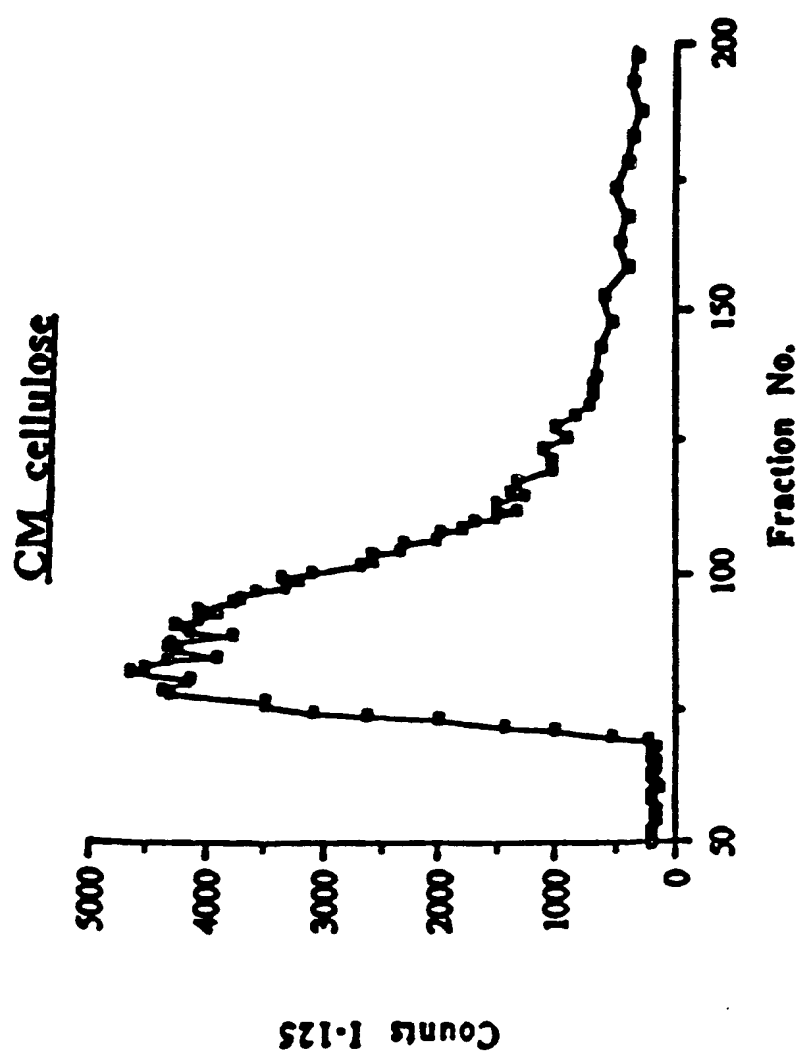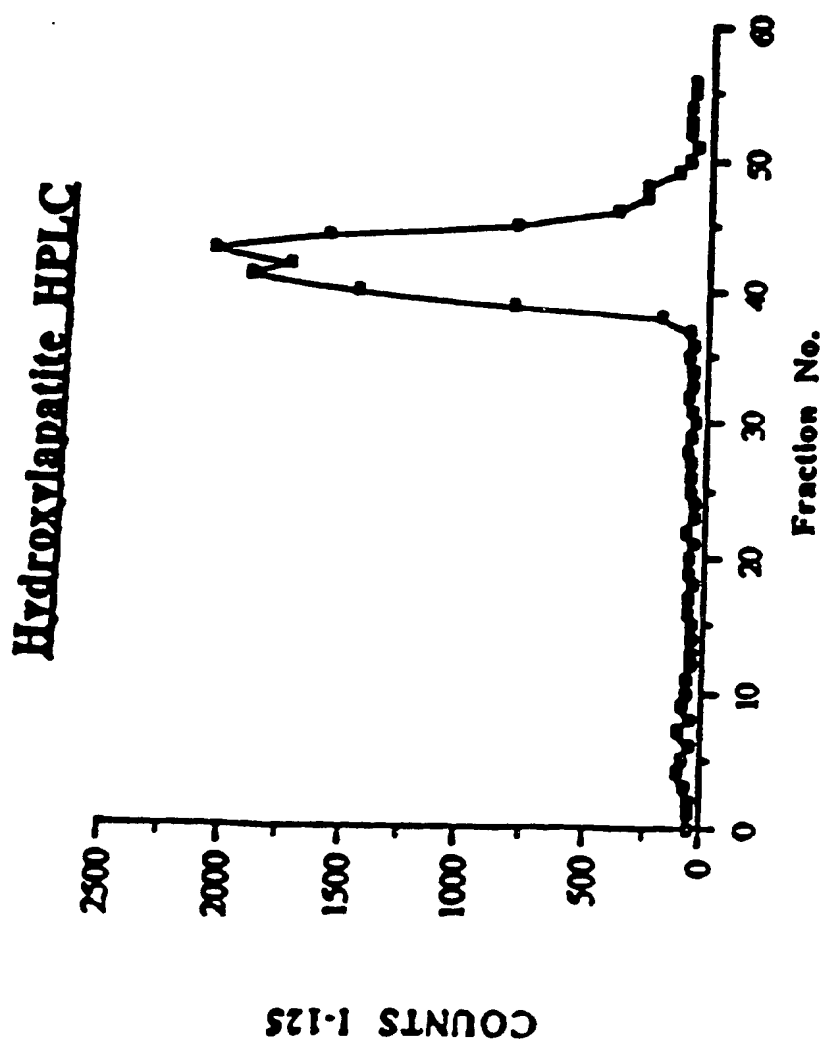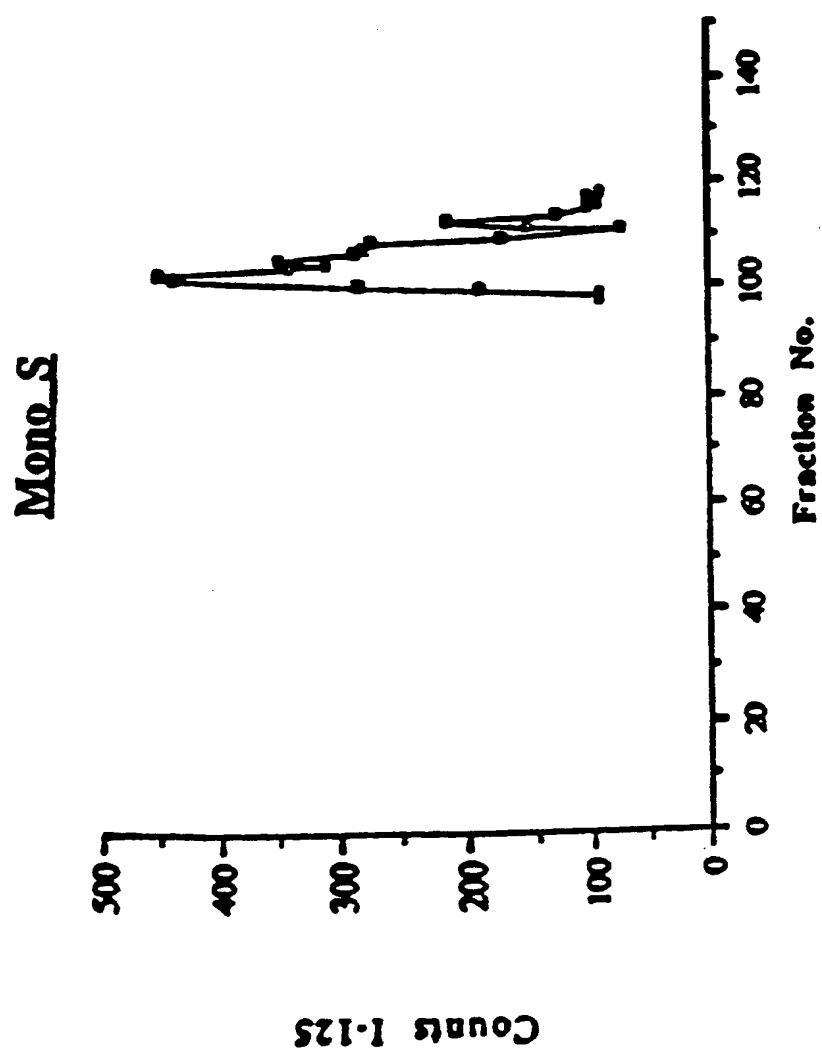
FIGURE 2
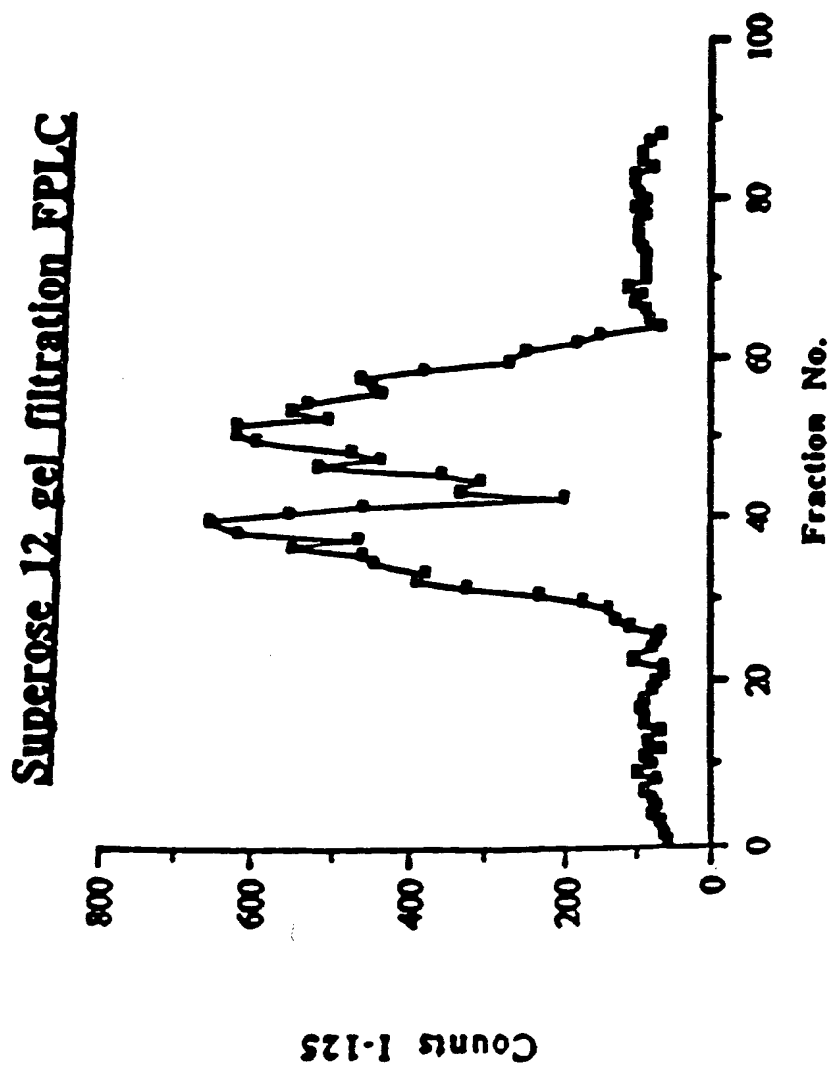
FIGURE 3

FIGURE 4

FIGURE 5

Reversed phase HPLC

FIGURE 6

FIGURE 7

FIGURE 8

FIGURE 9

N-terminus
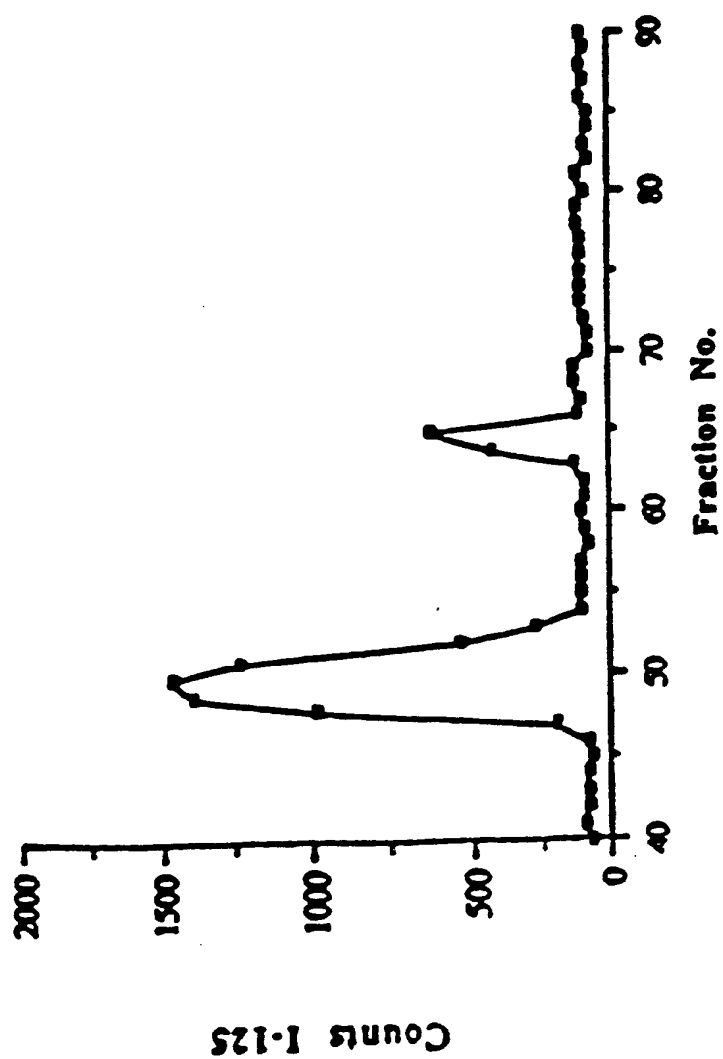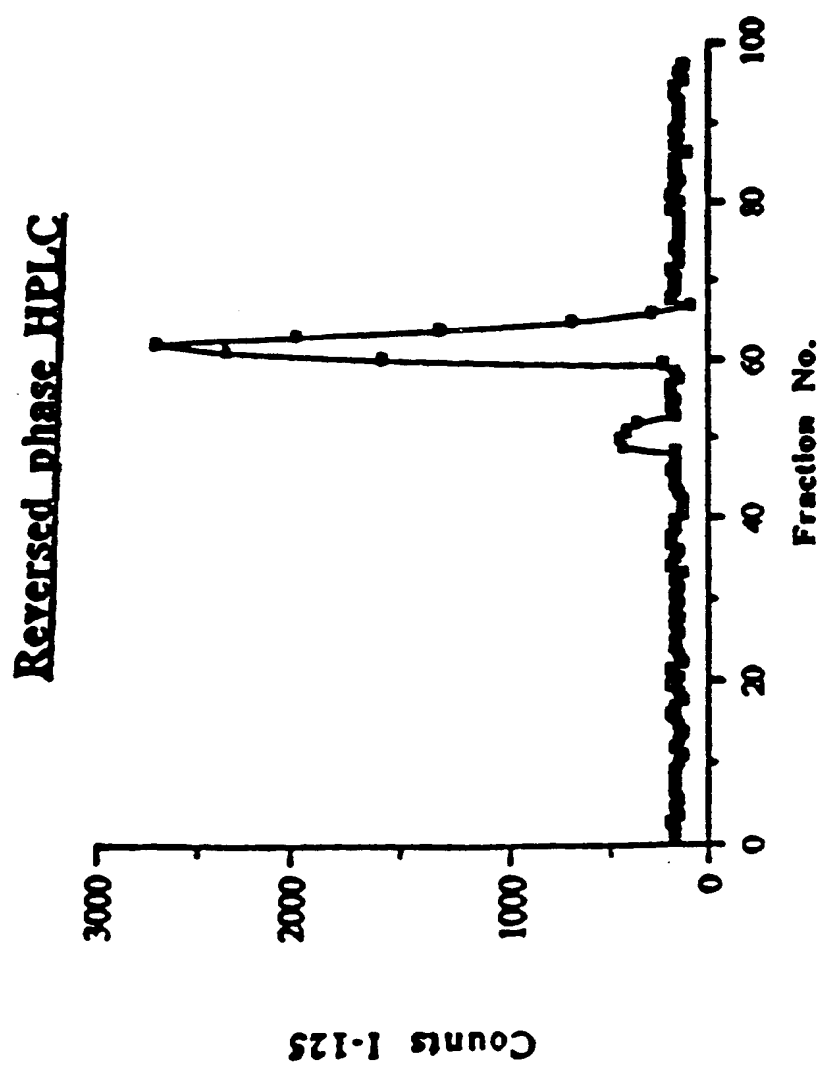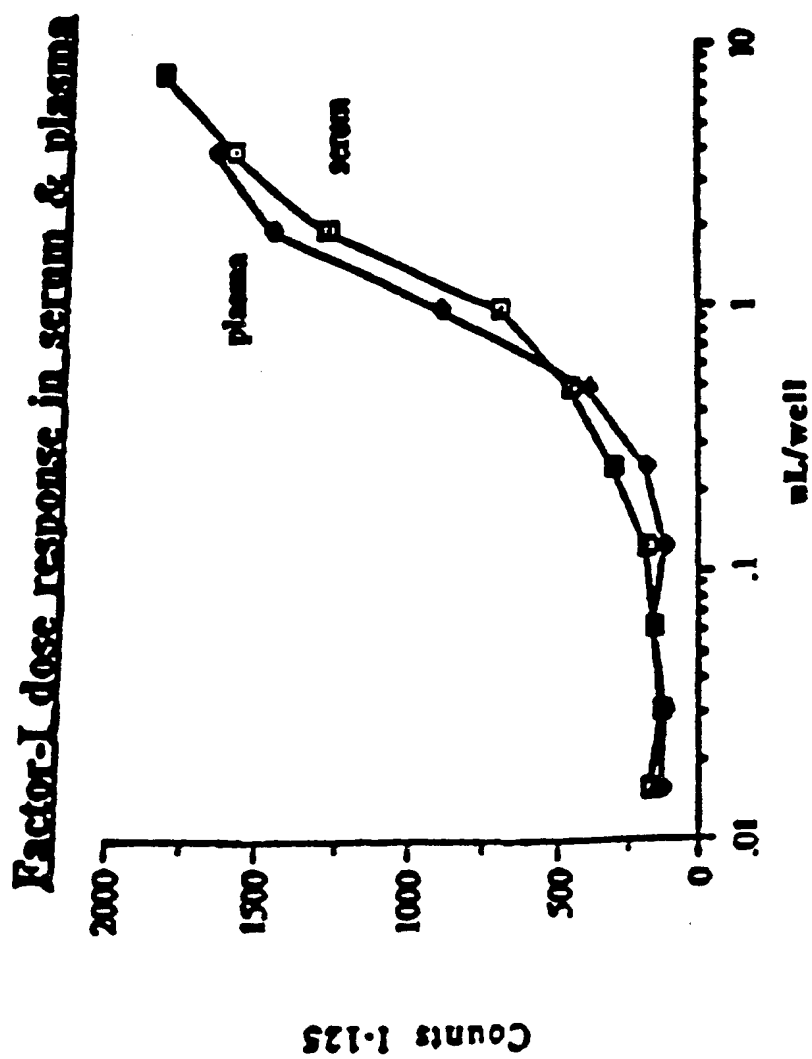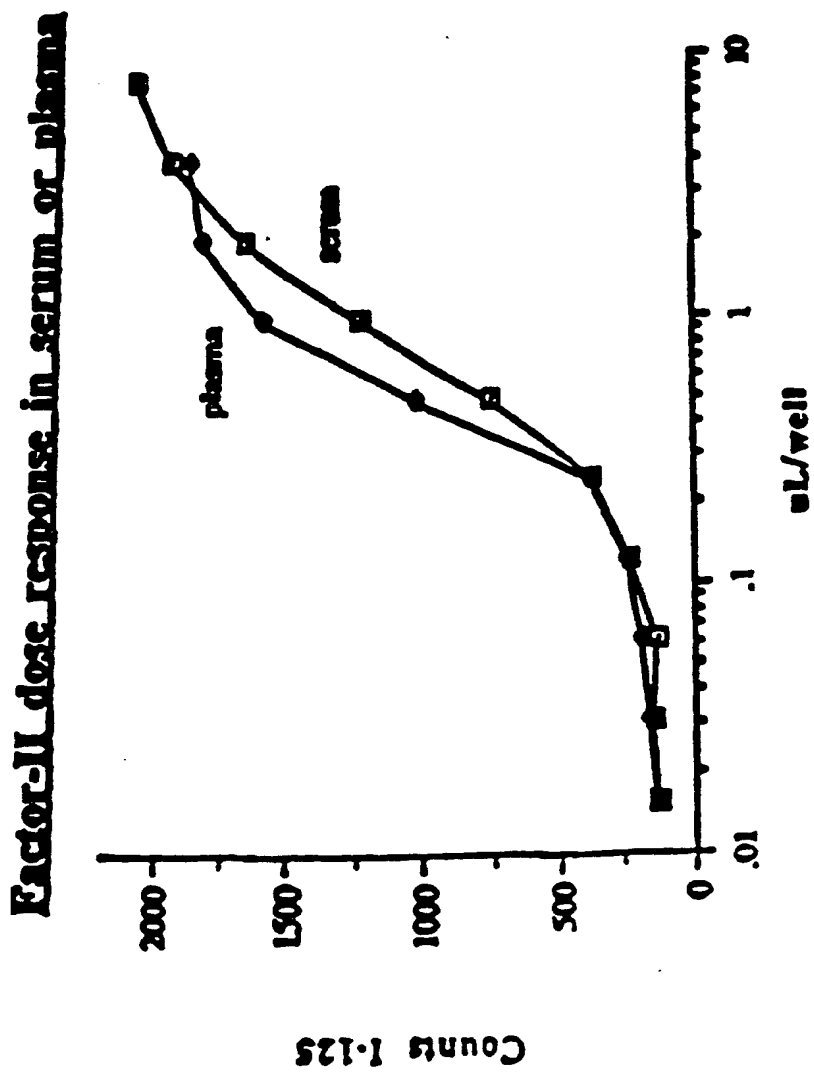
F K G D A N T E (SEQ ID NO: 1)

Trypsin peptides

KVR A S L A D E Y E Y M X K · (SEQ ID NO: 2)   HMG-1
KVR T E T S S S G L X L K · (SEQ ID NO: 3)   HMG-17
KVR K L G E M W A E (SEQ ID NO: 4)   HMG-2
KVR L G E K R A (SEQ ID NO: 5)
KVR I K S E H A G L S I G D T A K · (SEQ ID NO: 6)   HMG-1
KVR A S L A D E Y E Y M R K · (SEQ ID NO: 7)   HMG-2
KVR I K G E H P G L S I G D V A K · (SEQ ID NO: 8)   HMG-1
KVR M S E Y A F F V Q T X R · (SEQ ID NO: 9)
KVR S E H P G L S I G D T A K · (SEQ ID NO: 10)
KVR A G Y F A E X A R · (SEQ ID NO: 11)
KVR K L E F L X A K · (SEQ ID NO: 12)
KVR T T E M A S E O G A (SEQ ID NO: 13)   HMG-1
KVR A K E A L A A L K · (SEQ ID NO: 14)
KVR F V L Q A K K · (SEQ ID NO: 15)
KVR L G E M W (SEQ ID NO: 16)

Protease V8 peptides

E T O P D P G O I L K K V P M V I G A Y T (SEQ ID NO: 16)
E Y K C L K F K W F K K A T V M (SEQ ID NO: 17)
E A K Y F S K X D A (SEQ ID NO: 18)   LH-alpha
E X K F Y V P (SEQ ID NO: 19)
E L S F A S V R L P G C P P G V D P M V S F P V A L   LH-beta (SEQ ID N

Figure 10

A

GGF-I 01	FKGDANTE	(SEQ ID NO: 21)
GGF-I 02	ASLADEYEYHXK	(SEQ ID NO: 22)
GGF-I 03	TETSSGLXLK	(SEQ ID NO: 23)
GGF-I 07	ASLADEYEYHRK	(SEQ ID NO: 24)
GGF-I 11	AGYFAEXAR	(SEQ ID NO: 25)
GGF-I 13	TTEMASEQGA	(SEQ ID NO: 26)
GGF-I 14	AKEALAALK	(SEQ ID NO: 27)
GGF-I 15	FVLQAKK	(SEQ ID NO: 28)
GGF-I 17	ETQPDPGQILKKVPHVIGAYT	(SEQ ID NO: 29)
GGF-I 18	EYKCLKPKWKKATVH	(SEQ ID NO: 30)

B

GGF-I 20	EXKFYVP	(SEQ ID NO: 31)
GGF-I 12	KLEFLXAK	(SEQ ID NO: 32)

Figure 11

Trypsin peptides

GF-II 01	K/R　VHQVWAAK•	
GF-II 02	K/R　YIFFMEPEAXSSG	(SEQ ID NO: 45)
GF-II 03	K/R　LGAWGPPAFPVXY	(SEQ ID NO: 46)
GF-II 04	K/R　WFVVIEGK•	(SEQ ID NO: 47)
GF-II 05	K/R　ALAAAGYDVEK•	(SEQ ID NO: 48)
GF-II 06	K/R　LVLR•	Histone H1　(SEQ ID NO: 164)
GF-II 07	K/R　XXYPGQITSN	(SEQ ID NO: 165)
GF-II 08	K/R ASPVSVGSVQELVQR•	Trypsin　(SEQ ID NO: 166)
GF-II 09	K/R VCLLTVAALPPT	(SEQ ID NO: 49)
GF-II 10	K/R DLLLXV	(SEQ ID NO: 50)

Lysyl Endopeptidase-C peptides

GF-II 11	KVHQVWAAK•	
GF-II 12	KASLADSGEYMXK•	(SEQ ID NO: 51)
		(SEQ ID NO: 52)

Figure 12

A

GGF-II 01	VHQVWAAK	(SEQ ID NO: 45)
GGF-II 02	YIFFMEPEAXSSG	(SEQ ID NO: 46)
GGF-II 03	LGAWGPPAFPVXY	(SEQ ID NO: 47)
GGF-II 04	WFVVIEGK	(SEQ ID NO: 48)
GGF-II 08	ASPVSVGSVQELVQR	(SEQ ID NO: 49)
GGF-II 09	VCLLTVAALPPT	(SEQ ID NO: 50)
GGF-II 11	KVHQVWAAK	(SEQ ID NO: 51)
GGF-II 12	KASLADSGEYMXK	(SEQ ID NO: 52)

B   Novel Factor II Peptides - others

| GGF-II 10 | DLLLXV | (SEQ ID NO: 53) |

Comparison of BrdU-ELISA and (125 I)UdR counting method for the DNA synthesis assay in Schwann cell cultures
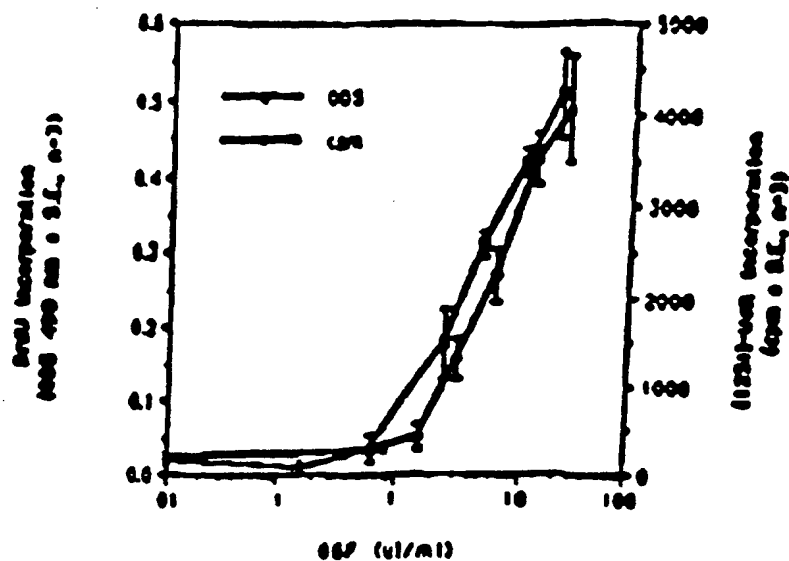
Fig. 13

Comparison of Br-UdR immunoreactivity and Br-UdR labelled cell number
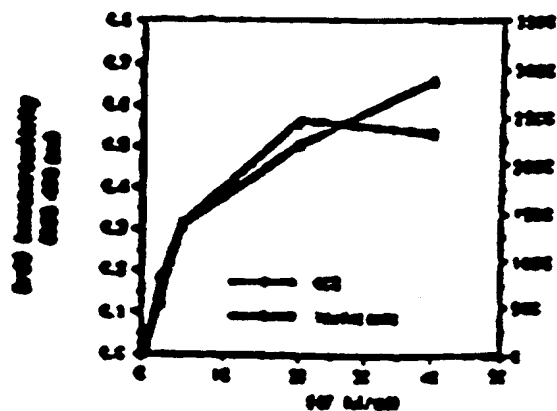
Fig. 14a
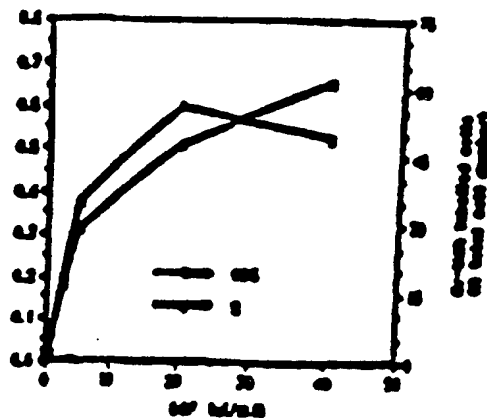
Fig. 14b.

Mitogenic response of rat sciatic nerve Schwann cell to GGFs
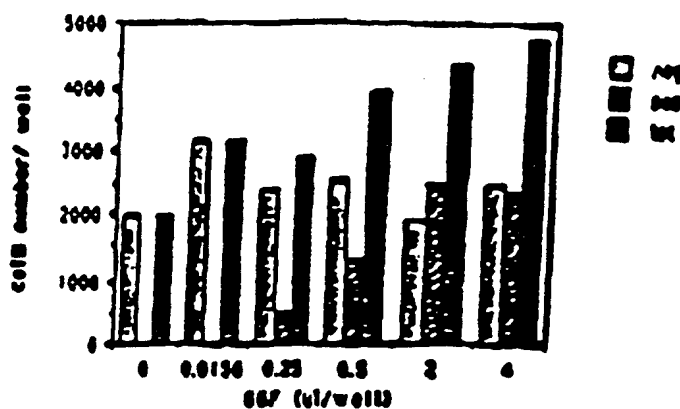
Fig. 15

DNA synthesis in rat sciatic nerve Schwann cells
and 3T3 fibroblasts in the presence of GGFs
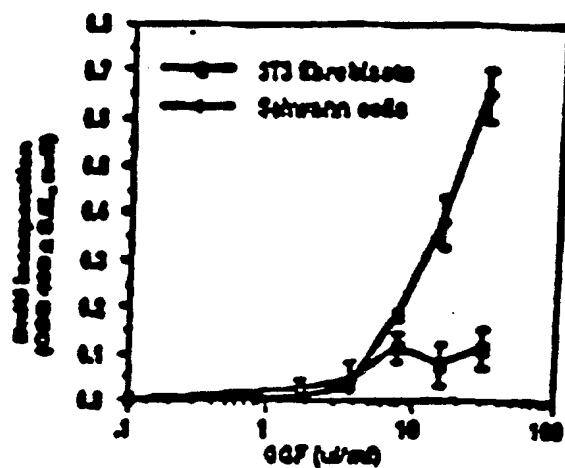
Fig. 16.

Mitogenic response of BHK 21 C13 cells to FCS and GGFs
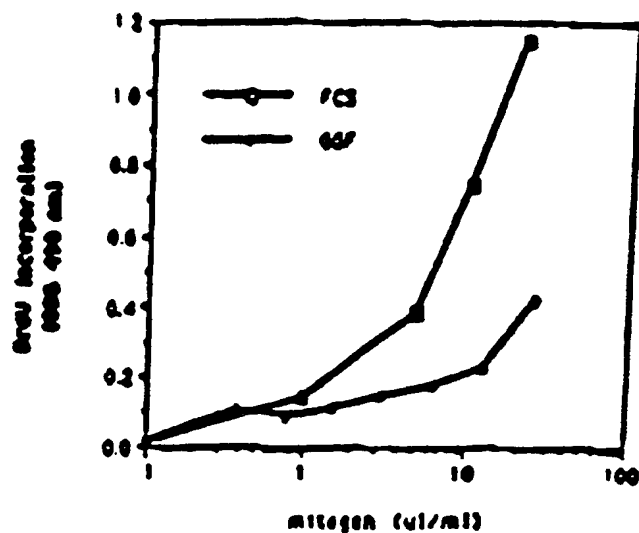
Fig. 17

Survival and proliferation of BHK21 C13 cell microcultures after 48 hours in presence of GGFs
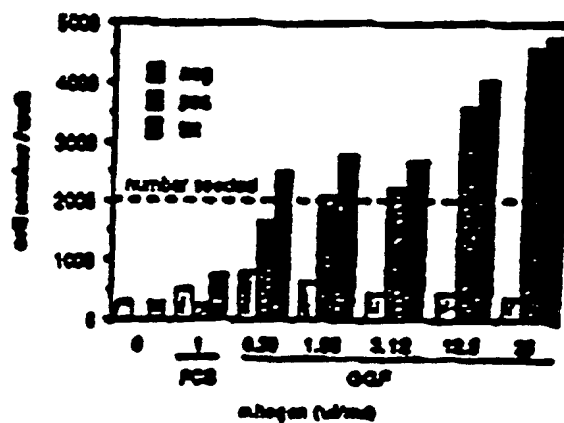
Fig. 18.

Mitogenic response of C6 cells to FCS
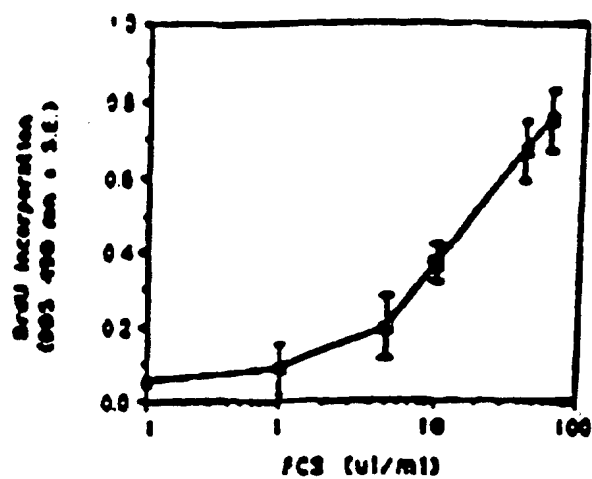
Fig 19.

Mitogenic response of C6 cells to aFGF and GGFs
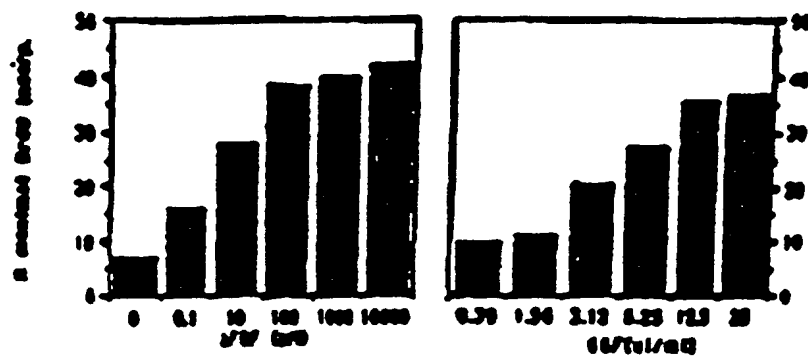
Fig 20

FIGURE 21

DEGENERATE OLIGONUCLEOTIDE PROBES FOR FACTOR I AND FACTOR II

Oligo	Sequence	Peptide	
535	TTYAARGGNGAYGCNCAYAC!	GGFI-1	(SEQ ID NO: 54)
536	CATRTAYTCRTAYTCRTCNGC!	GGFI-2	(SEQ ID NO: 55)
537	TGYTCNGANGCCATYTCNGT!	GGFI-13	(SEQ ID NO: 56)
538	TGYTCRCTNGCCATYTCNGT!	GGFI-13	(SEQ ID NO: 57)
539	CCDATNACCATNGGNACYTT!	GGFI-17	(SEQ ID NO: 58)
540	GCNGCCCANACYTGRTGNAC!	GGFII-1	(SEQ ID NO: 59)
541	GCYTCNGGYTCCATRAARAA!	GGFII-2	(SEQ ID NO: 60)
542	CCYTCDATNACNACRAACCA!	GGFII-4	(SEQ ID NO: 61)
543	TCNGCRAARTANCCNGC!	GGFI-11	(SEQ ID NO: 62)
544	GCNGCNAGNGCYTCYTTNGC!	GGFI-14	(SEQ ID NO: 63)
545	GCNGCYAANGCYTCYTTNGC!	GGFI-14	(SEQ ID NO: 64)
546	TTYTTNGCYTGNAGNACRAA!	GGFI-15	(SEQ ID NO: 65)
551	TTYTTNGCYTGYAANACRAA!	GGFI-15	(SEQ ID NO: 66)
568	TGNACNAGYTCYTGNAC!	GGFII-8	(SEQ ID NO: 67)
569	TGNACYAAYTCYTGNAC!	GGFII-8	(SEQ ID NO: 68)
609	CATRTAYTCNCCNGARTCNGC!	GGFII-13	(SEQ ID NO: 69)
610	CATRTAYTCNCCRCTRTCNGC!	GGFII-13	(SEQ ID NO: 70)
649	NGARTCNGCYAANGANGCYTT!	GGFII-13	(SEQ ID NO: 71)
650	NGARTCNGCNAGNGANGCYTT!	GGFII-13	(SEQ ID NO: 72)
651	RCTRTCNGCYAANGANGCYTT!	GGFII-13	(SEQ ID NO: 73)
652	RCTRTCNGCNAGNGANGCYTT!	GGFII-13	(SEQ ID NO: 74)
653	NGARTCNGCYAARCTNGCYTT!	GGFII-13	(SEQ ID NO: 75)
654	NGARTCNGCNAGRCTNGCYTT!	GGFII-13	(SEQ ID NO: 76)
655	RCTRTCNGCYAARCTNGCYTT!	GGFII-13	(SEQ ID NO: 78)
656	RCTRCTNGCNAGRCTNGCYTT!	GGFII-13	(SEQ ID NO: 79)
659	ACNACNGARATGGCTGGNGA!	GGFI-13	(SEQ ID NO: 80)
660	ACNACNGARATGGCAGYNGA!	GGFI-13	(SEQ ID NO: 81)
661	CAYCARGTNTGGGCNGCNAA!	GGFII-1	(SEQ ID NO: 82)
662	TTYGTNGTNATBGARGGNAA!	GGFII-4	(SEQ ID NO: 83)
663	AARGGNGAYGCNCAYACNGA!	GGFI-1	(SEQ ID NO: 84)
664	GARGCNYTNGCNGCNTNAA!	GGFI-14	(SEQ ID NO: 85)
665	GTNGGNTCNGTNCARGANTT!	GGFII-8	(SEQ ID NO: 86)
666	GTNGGNAGYGTNCARGANTT!	GGFII-8	(SEQ ID NO: 87)
694	NACYTTYTTNARRATYTGNCC!	GGFI-17	(SEQ ID NO: 88)

Putative Serine factor II Gene Sequence

TCTAAACTACACAGCACTCTATTTCATCATCATCGTACTTCTTCAAATATACTTAAGCCCTTTACTCCTCATCTTCTTACCAATCACAACTTCAAT   100

TACCAAACCTCACTACCTCATTCTCCACCAATAAATTCCAAATCATCCCAACTACCAATCACACTCCTCTCCCAACTACCCATTCTCAAGTCA   200

AACACTACCACATCCCTACTCCTACCTATTTCTCCAATCTCAAACCATCATCCATCACCACTCATCAATCCCCAACGTCTTCAAATCTCAT   300

TTTCACACAATAAAATCATCAACACAAATCTCTATCATTCAAATATCATATCCCATCCTCCTCTAAAACCTCTTCAATCCCATTAACTACAAATAACCCTATCAT   400

ATATATATAGATTATTT

(SEQ ID NO: 89)

FIGURE 22

PCR PRIMERS FOR FACTOR I AND FACTOR II   **FIGURE 23**

Degenerate PCR primers

Oligo	Sequence	Peptide	
657	CCGAATTCTGCAGGARACNCARCCNGAYCCNGG!	GGFI-17	(SEQ ID NO: 90)
658	AAGGATCCTGCAGNGTRTANGCNCCRATNACCATNGG!	GGFI-17	(SEQ ID NO: 91)
667	CCGAATTCTGCAGGCNGAYTCNGGNGARTAYATG!	GGFII-13	(SEQ ID NO: 92)
668	CCGAATTCTGCAGGCNGAYATYGGNGARTAYAT!	GGFII-13	(SEQ ID NO: 93)
669	AAGGATCCTGCAGNNNCATRTAYTCNCCNGARTC!	GGFII-13	(SEQ ID NO: 94)
670	AAGGATCCTGCAGNNNCATRTAYTCNCCRRTRTC!	GGFII-13	(SEQ ID NO: 95)
671	CCGAATTCTGCAGCAYCARGTNTGGGCNGCNAA!	GGFII-1	(SEQ ID NO: 96)
672	CCGAATTCTGCAGATHTTYTTYATGGARCCNGARG!	GGFII-2	(SEQ ID NO: 97)
673	CCGAATTCTGCAGGGGGNCCNCCNGCNTTYCCNGT!	GGFII-3	(SEQ ID NO: 98)
674	CCGAATTCTGCAGTGGTTYGTNGTNATHGARGG!	GGFII-4	(SEQ ID NO: 99)
677	AAGGATCCTGCAGYTTNGCNGCCCANACYTGRTG!	GGFII-1	(SEQ ID NO: 100)
678	AAGGATCCTGCAGGCYTCNGGYTCCATRAARAA!	GGFII-2	(SEQ ID NO: 101)
679	AAGGATCCTGCAGACNGGRAANGCNGGNGGNCC!	GGFII-3	(SEQ ID NO: 102)
680	AAGGATCCTGCAGYTTNCCYTCDATNACNACRAAC!	GGFII-4	(SEQ ID NO: 103)
681	CATRTAYTCRTAYTCTCNGCAAGGATCCTGCAG!	GGFI-2	(SEQ ID NO: 104)
682	CCGAATTCTGCAGAARGGNGAYGCNCAYACNGA!	GGFI-1	(SEQ ID NO: 105)
683	GCNGCYAANGCYRCYTTNGCAAGGATCCTGCAG!	GGFI-14	(SEQ ID NO: 106)
684	GCNGCNAGNGCYTCYTTNGCAAGGATCCTGCAG!	GGFI-14	(SEQ ID NO: 107)
685	TCNGCRAARTANCCNGCAAGGATCCTGCAG!	GGFII-1	(SEQ ID NO: 108)

Unique PCR primers for Factor II

Oligo	Sequence	Comment	
711	CATCGATCTGCAGGCTGATTCTGGAGAATATATGTGCA!	3' RACE	(SEQ ID NO: 109)
712	AAGGATCCTGCAGCCACATCTCGAGTCGACATCGATT!	3' RACE	(SEQ ID NO: 110)
713	CCGAATTCTGCAGTGATCAGCAAACTAGGAAATGACA!	3' RACE	(SEQ ID NO: 111)
721	CATCGATCTGCAGCCTAGTTTGCTGATCACTTTGCAC!	5' RACE	(SEQ ID NO: 112)
722	AAGGATCCTGCAGTATATTCTCCAGAATCAGCCAGTG!	5' RACE; ANCHORED	(SEQ ID NO: 11:)
725	AAGGATCCTGCAGGCACGCAGTAGGCATCTCTTA!	EXON A	(SEQ ID NO: 114)
726	CCGAATTCTGCAGCAGAACTTCGCATTAGCAAAGC!	EXON A	(SEQ ID NO: 115)
771	CATCCCGGGATGAAGAGTCAGGAGTCTGTGGCA!	EXONS B+A	(SEQ ID NO: 116)
772	ATACCCGGGCTGCAGACAATGAGATTTCACACACCTGCG!		(SEQ ID NO: 117)
773	AAGGATCCTGCAGTTTGGAACCTGCCACAGACTCCT!	ANCHORED	(SEQ ID NO: 118)
776	ATACCCGGGCTGCAGATGAGATTTCACACACCTGCCTGA!	EXONS B+A	(SEQ ID NO: 119)

Summary of contiguous GGF-II cDNA structures and sequences
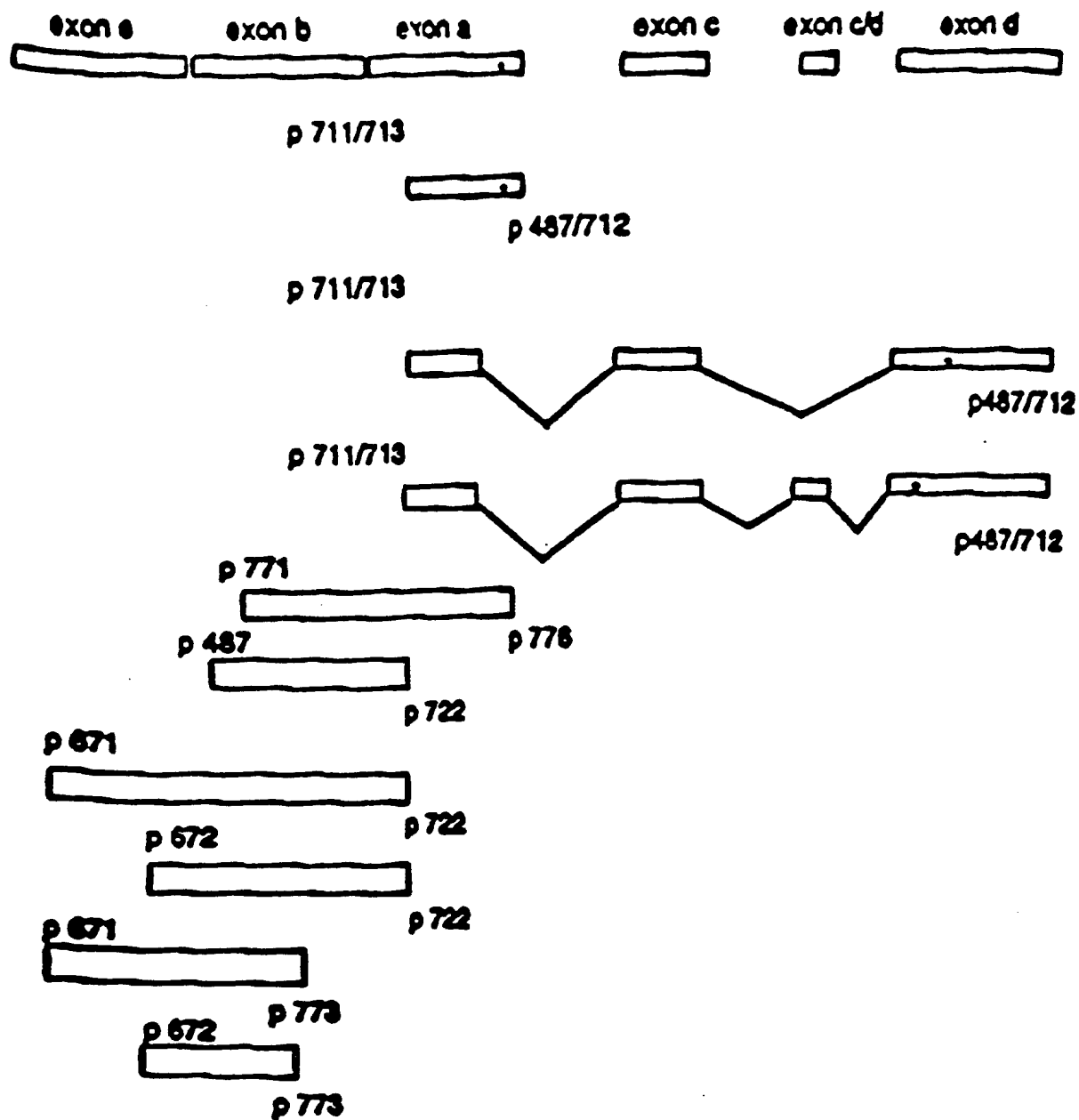
FIGURE 24

FIGURE 25
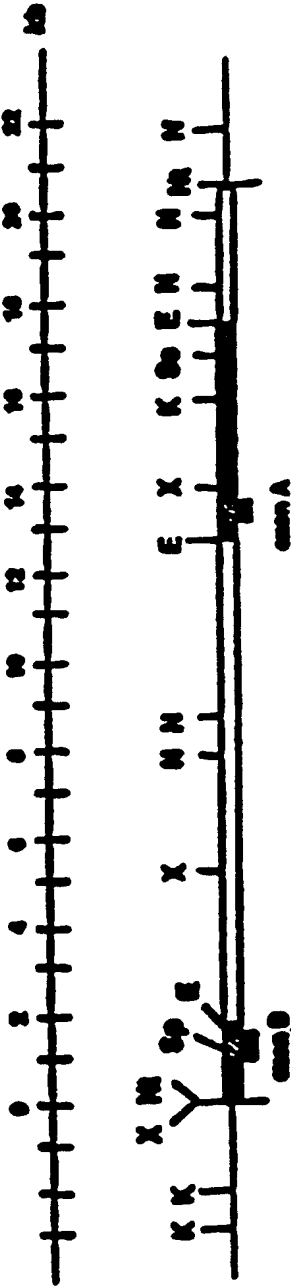
Alternative gene products of putative bovine GGF-2
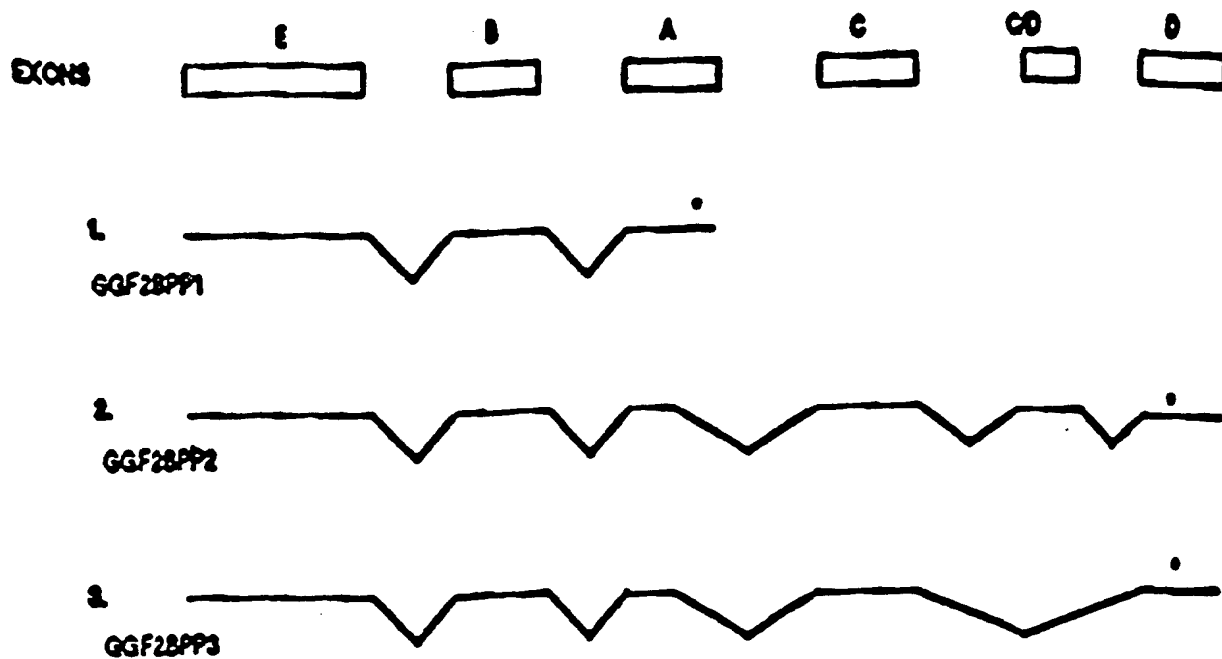
FIGURE 26

OGP-II peptides identified in deduced amino acid sequences
of putative bovine OGP-II proteins

Peptide	Pos.	Sequence match	
II-1		VHQVWAAK	
	1:	HQVWAAK AAGLK	(SEQ ID NO: 120)
II-10		DLLLKV	
	14:	GGLKK dslltv RLGAW	(SEQ ID NO: 121)
II-03		LGAWGPPAFPVKY	(SEQ ID NO: 122)
	21:	LLTVR lgavghpafpecg RLKZD	(SEQ ID NO: 123)
II-02		YIFFKEPEAKSSG	(SEQ ID NO: 124)
	41:	KEDSR YIFTHEPEANSSG GPGRL	(SEQ ID NO: 125)
II-6		LVLR	
	103:	VAGSK LVLR CETSS	(SEQ ID NO: 126)
I-18		EYKCLKFKWFKKATVK	(SEQ ID NO: 127)
	112:	CETSS eysslkfkvfkngsel SRKWK	(SEQ ID NO: 128)
II-12		KASLADSGEYKCK	(SEQ ID NO: 129)
	151:	ELRIS KASLADSGEYHCK VISKL	(SEQ ID NO: 130)
I-07		ASLADEYEYKCK	(SEQ ID NO: 131)
	152:	LRISK asladsgeyack VISKL	(SEQ ID NO: 132)

FIGURE 27

(SEQ ID NO: 133)

FIGURE 28A

Nucleotide sequence and deduced amino acid sequence of ORF-A

(SEQ ID NO: 134)

FIGURE 28B

(SEQ ID NO: 135)

FIGURE 28C

FIGURE 29

Figure - 30

FIGURE 31   (1 of 8)

CODING SEGMENTS OF GLIAL GROWTH FACTOR/HEREGU, K-GGF2

CODING SEGMENT P: (SEQ ID NO: 136)

```
AGTTTCCCCCCCCAACTTGTCGGAACTCTGGGCTCGCGCGCAGGGCAGGAGCGGAGCCGC  60

GGCGGCTGCCCAGGCGATGCGAGCGCGCGGCCGGACGGTAATCGCCTCTCCGTCCTCGGGC  120

TGCGAGCGGCGCCGGACCGAGGCAGCGACAGGAGCGGACCGCGGCGCGGAACCGAGGACTCC  180

CCAGCCGCGCGCCCAGCAGGAGCCACCCCGCGAGTCGTGCGACCCCGACGGAGCGCCCGCC  240

AGTCCCAGGTGGCCCCGGACCGCACGTTCCGTCCCCGCGCTCCCCGCCCGGCGACAGGAGAC  300

GCTCCCCCCCACGCCGCGCGCGCGCCTCGGCCCCGGTCGCTGGCCCGCCTCCACTCCGGGGAC  360
            |||| |||||||| || ||| |||| || | |  || ||| |||||
            cgcgagcgcctcagcgcggccgctcgctctc..ccgctcgaggac

AAACTTTTCCCGAAGCCGATCCCAGCCCTCGGACCCAAACTTGTCGCGCGTCGCCTTCGC  420
|||||||||||| ||| ||||||| ||||| ||||| ||                |||||| |||
aaacttttcccaaacccgatccgagcccttggaccaaa..........ctcgcctgcgc

                    M  S  E  R  R  R  G
CGGCAGCCGTCCGCGCAGAGCCGTGCACTTCTCGGGCGAGATGTCGGAGCGCAGAGAAGGC  480
|| ||||||||||||| |||||| | | |||| |||||||||| ||||||| |||||||
cgagagccgtccgcgtagagcgctc.cgtctccggcgagatgtccgagcgcaaagaaggc
                                                        K

K  G  K  G  K  G  G  K  K  D  R  G  S  G  K  K  P  V  P  A
AAAGGCAAGGGGAAGGGCGGCAAGAAGGACCGAGGCTCCGGGAAGAAGCCCGTGCCCGCC  540
| |||||| ||||||||   |||||||| |||||||||||| |||||||| | | |||||
agaggcaaagggaagggcaagaagaaggagcgaggctccggcaagaagccggagtccgcg
R                   K           E                         E  S

A  G  G  P  S  P  A
GCTGGCGGCCCGAGCCCAG  559
|| |||| ||| |||||||||
gcgggcagccagagcccag
```

CODING SEGMENT E: (SEQ ID NO: 137)

```
CCCATCATGTGTGGCGGCGAAAGCCGCGGGGCTTGAAGAAGCACTCGCTGCTCACCGTGC  60
    H  Q  V  W  A  A  K  A  G  G  L  K  K  D  S  L  L  T  V  E

GCCTGGGCGCGTGGGGCCACCCCGCCTTCCCCTCGTGCGGGCGCCTCAAGGAGGACAGCA  120
  L  G  A  W  G  H  P  A  F  P  S  C  G  R  L  K  E  D  S  R

GGTACATCTTCTTCATGGAGCCCGAGGCCAACAGCAGCGGCGTGCCCGGCCGCCTTCCCA  180
  Y  I  F  F  M  E  P  E  A  N  S  S  G  C  P  G  R  L  P  S

GCCTCCTTCCCCCTCTCCGAGACGGGCCGAAACCTCAAGAAGGAGGTCAGCCCGGTGCTG  240
  L  L  P  P  S  R  D  G  P  E  P  Q  E  G  G  Q  P  G  A  V

TCCAAGGTCCC  251
  S  K  V
```

FIGURE 31 (CONT.)

CODING SEGMENT B: (SEQ ID NO: 138)

```
    L  P  P  R  L  K  E  N  K  S  Q  E  S  V  A  G  S  K  L  V
   CCTTGCCTCCCCGCTTGAAAGAGATGAAGAGTCAGGAGTCTGTGGCAGGTTCCAAACTAG   60
   |||||||||||||||| |||||||||||||||| || ||||| || |  ||||||||||||||||
   ccttgcctccccgattgaaagagatgaaaagccaggaatcggctgcaggttccaaactag
                   Q                              A
```

```
    L  R  C  E  T  S  S  E  Y  S  S  L  K  F  K  V  F  K  N  G
   TGCTTCGGTGCGAGACCAGTTCTGAATACTCCTCTCTCAAGTTCAAGTGGTTCAAGAATG   120
   |  |||||||| || |||||||||||||||||||||||||  ||||||||||||||||||||
   tccttcggtgtgaaaccagttctgaatactcctctctcagattcaagtggttcaagaatg
                                                R
```

```
    S  E  L  S  R  K  N  P  Q  N  I  K  I  Q  K  R  P  G
   GGAGTGAATTAAGCCGAAAGAACAACCACAAAACATCAAGATACAGAAAGGCCGGG   178
   ||| |||||| || | ||||| |||||||||||||||| |||||||||| |||| ||| ||
   ggaatgaattgaatcgaaaaaactaaccacaaaatatcaagatacaaaaaagccagg
     N         N                                         K
```

CODING SEGMENT A: (SEQ ID NO: 139)

```
    K  S  E  L  R  I  S  K  A  S  L  A  D  S  G  E  Y  N  C  K
   GAAGTCAGAACTTCGCATTAGCAAAGCGTCACTGGCTGATTCTGGAGAATATATGTGCAA   60
   ||||||||||||||||||||||||||| |||||| ||||||||||||||||| |||||||||||
   gaagtcagaacttcgcattaacaaagcatcactggctgattctggagagtatatgtgcaa
                             N
```

```
    V  I  S  K  L  G  N  D  S  A  S  A  N  I  -  I  V  E  S  N
   AGTGATCAGCAAACTAGGAAATGACAGTGCCTCTGCCAACATCACCATTGTGGAGTCAAA   120
   ||||||||||||||| |||||||||||||||||||||||||| |||||||| |||||| |||||
   agtgatcagcaaattaggaaatgacagtgcctctgccaatatcaccatcgtggaatcaaa
```

```
       A
       CG   122
       ||
       cg
```

CODING SEGMENT A':   (SEQ ID NO: 140)

```
   TCTAAAACTACAGAGACTGTATTTTCATGATCATCATAGTTCTGTGAAATATACTTAAAC   60
```

```
                       K  S  E  L  R  I  S  K  A  S  L  A  D
   CGCTTTGGTCCTGATCTTGTAGGAAGTCAGAACTTCGCATTAGCAAAGCGTCACTGGCTG   120
```

```
     S  G  E  Y  N  C  K  V  I  S  K  L  G  N  D  S  A  S  A  N
   ATTCTGGAGAATATATGTGCAAAGTGATCAGCAAACTAGGAAATGACAGTGCCTCTGCCA   180
```

```
     I  T  I  V  E  S  N  G  K  R  C  L  L  R  A  I  S  Q  S  L
   ACATCACCATTGTGGAGTCAAACGGTAAGAGATGCCTACTGCGTGCTATTTCTCAGTCTC   240
```

```
     R  G  ?  ?  K  ?  C  ?  N  ?  ?  *
   TAAGAGGA?GA??AAG??AT????????CACA???AATCAC?CAG????????AAA???CA?   ???
```

FIGURE 31 (CONT.) (3   8)

TGTCAACAAATAAAAATCATGAAAGGAAAACTCTATGTTTGAAATATCTTATGGGTCCTC 360

CTGTAAAGCTCTTCACTCCATAAGGTCAAATAGACCTGAAATATATATAGATTATTT 417

CODING SEGMENT G: (SEQ ID NO: 141)

```
   E  I  T  T  G  M  P  A  S  T  E  T  A  Y  V  S  S  E  S  P  i
   AGATCACCACTGGCATGCCAGCCTCAACTGAGACAGCGTATGTGTCTTCAGAGTCTCCCA  60
   |||||||  ||||||  ||||||||||||  |||  |||||||||||||||||||||||||
   agatcatcactggtatgccagcctcaactgaaggagcatatgtgtcttcagagtctccca
            I                           G

   R  I  S  V  S  T  E  G  T  N  T  S  S  S
   TTAGAATATCAGTATCAACAGAAGGAACAAATACTTCTTCAT  102
   ||||||||||||||||||| |||||||||  ||||||||||||
   ttagaatatcagtatccacagaaggagcaaatacttcttcat
                        A
```

CODING SEGMENT C: (SEQ ID NO: 160)

```
   T  S  T  S  T  A  G  T  S  N  L  V  K  C  A  E  K  E  K  i
   CCACATCCACATCTACAGCTGGGACAAGCCATCTTGTCAAGTGTGCAGAGAAGGAGAAA  60
   | ||||||  ||||||  ||  ||||||||||||||||||||  ||  ||||||  |||||||||||||||
   ctacatctacatccaccactgggacaagccatcttgtaaaatgtgcggagaaggagaaa
            T

   F  C  V  N  G  G  E  C  F  M  V  K  D  L  S  N  P  S  R  i
   CTTTCTGTGTGAATGGAGGCGAGTGCTTCATGGTGAAAGACCTTTCAAATCCCTCAAGAT  120
   |||||||||||||||||||  ||||||||||||||||||||||||||||||||  |||||  ||||
   ctttctgtgtgaatggagggggagtgcttcatggtgaaagacctttcaaaccctcgagat

   L  C
   ACTTGTGC  128
   ||||||||
   acttgtgc
```

FIGURE 31 (CONT.) (7 of 8)

CODING SEGMENT C/D: (SEQ ID NO: 142)

```
  K   C   Q   P   G   F   T   G   A   R   C   T   E   N   V   P   N   K   V   Q
AAGTGCCAACCTGGATTCACTGGAGCGAGATGTACTGAGAATGTGCCCATGAAAGTCCAA  60
|||||||||||||||||||||||||||| |||||||||||||||||||||||||||||||
aagtgccaacctggattcactggagcaagatgtactgagaatgtgcccatgaaagtccaa
```

```
  T   Q   E
ACCCAAGAA  69
| |||||||
aaccaagaa
N
```

CODING SEGMENT C/D': (SEQ ID NO: 143)

```
  K   C   P   N   E   F   T   G   D   R   C   Q   N   Y   V   N   A   S   F   Y
AAGTGCCCAAATGAGTTTACTGGTGATCGCTGCCAAAACTACGTAATGGCCAGCTTCTAC  60
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
aagtgcccaaatgagtttactggtgatcgctgccaaaactacgtaatggccagcttctac
```

CODING SEGMENT D: (SEQ ID NO: 144)

```
  S   T   S   T   P   F   L   S   L   P   E   *
AGTACGTCCACTCCCTTTCTGTCTCTGCCTGAATAG  36
||||||||||||||||||||||||||||||||||||
agtacgtccactccctttctgtctctgcctgaatag
```

CODING SEGMENT D': (SEQ ID NO: 145)

```
  K   E   L   G   I   E   F   M   E
aagcatcttgggattgaatttatggag  27
```

FIGURE 31 (CONT.)   5 -- 8)

CODING SEGMENT N: (SEQ ID NO: 146)

```
     K  A  E  E  L  Y  Q  K  R  V  L  T  I  T  G  I  C  I  A  L
     AAAGCGGAGGAGCTCTACCAGAAGAGAGTGCTCACCATTACCGGCATTTgCATCGCGCTG   60
     || ||||||||||| ||||||||||||||||||| |||||| |||||||| |||||||| ||
     aaggcggaggagctgtaccagaagagagtgctgaccataaccggcatctgcatcgccctc

     L  V  V  G  I  M  C  V  V  V  Y  C  K  T  K  K  Q  R  K  K
     CTCGTGGTTGGCATCATGTGTGTGGTGGTCTACTGCAAAACCAAGAAACAACGGAAAAAg  120
     || |||||| |||||||||||||||||||||||| ||||||||||||||| ||||||||||
     cttgtggtcggcatcatgtgtgtggtggcctactgcaaaaccaagaaacagcggaaaaag
                              A

     L  I  D  R  L  R  Q  S  L  R  S  E  R  N  T  K  K  K  V  A
     CTTCATGACCGGCTTCGGCAGAgCCTTCGGTCTGAAAGAAACACCATGATGAACGTAGCC  180
     || ||||||||| ||||||||||||| |||||||||| ||||||| ||||||| | |||
     ctgcatgaccgtcttcggcagagccttcggtctgaacgaaacaatatgatgaacattgcc
                              N                       I

     N  G  P  K  R  P  N  P  P  P  E  N  V  Q  L  V  N  Q  Y  V
     AACCGgCCCCCACCACCCCCAATCCGCCCCCCCGAGAACGTGCAGCTGGTGAATCAATACGTA  240
     || ||||| ||||| || || || |||||||| || ||||||||||||||| ||||| ||
     aatgggcctcaccatcctaacccacccccgagaatgtccagctggtgaatcaatacgta

     S  K  N  V  I  S  S  E  R  I  V  E  R  E  A  R  S  S  F  S
     TCTAAAAATGTCATCTCTAGCGAGCATATTGTTGAGAGAGAGGCGGAGAGCTCTTTTTCC  300
     |||||||| |||||||| ||||||||| || ||||||||||||||||| || |||| || ||||||
     tctaaaaacgtcatctccagtgagcatattgttgagagagaagcagagacatccttttcc
                                                      T

     T  S  N  Y  T  S  T  A  R  R  S  T  T  V  F  Q  T  P  S  N
     ACCAGTCACTACACTTCGACAGCTCATCATTCCACTACTGTCACTCAGACTCCCAGTCAC  360
     ||||||||||| ||||| |||||| |||||| |||||||||||||||||| ||||||| || |||
     accagtcactatacttccacagcccatcactccactactgtcacccagactcctagccac

     S  W  S  N  G  K  T  R  S  I  I  S  R  S  N  S  V  I  V  N
     AGCTGGAGCAATGGACACACTGAAAGCATCATTTCGGAAAGCCACTCTGTCATCGTGATC  420
     |||||||||| ||||||||||||||||||||||| |||| ||||||||||||||||||||
     agctggagcaacggacacactgaaagcatcctttccgaaagccactctgtaatcgtgatc
                                       L

     S  S  V  E  N  S  R  N  S  S  P  T  G  G  P  R  G  R  L  N
     TCATCCGTAGAAAACAGTAGGCACAGCAGCCCGACTGGGGGGCCCGAGAGGACGTCTCANF  480
     ||||||||||||| |||||| |||||||||||||||||||| |||||||||| |||||||| |||
     tcatccgtagaaaacagtaggcacagcagcccaactgggggcccagaggacgtcttaat

     G  L  G  G  P  R  E  C  N  S  F  L  R  H  A  R  E  T  P  D
     GGCTTGGGAGGCCCTCGTGAATGTAACAGCTTCCTCAGGCATGCCAGAGAAACCCCTGAC  540
     ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
     ggcacaggaggccctcgtgaatgtaacagcttcctcaggcatgccagagaaacccctgat
       T

     S  Y  R  D  S  P  H  S  E  R
     TCCTACCGAGACTCTCCTCATAGTGAAAG  569
     |||!|:|||||| !||||||||'||||||||
     tcctaccgagac:::::::catagtgaaag
```

FIGURE 31 (CONT.)

CODING SEGMENT K: (SEQ ID NO: 161)

```
ACATAACCTTATAGCTGAGCTAAGGAGAAACAAGGCCCACAGATCCAAATGCATGCAGAT
 H  N  L  I  A  E  L  R  R  N  K  A  N  R  S  K  C  N  Q  I

CCAGCTTTCCGCAACTCATCTTAGAGCTTCTTCCATTCCCCATTGGGCTTCATTCTCTAA
 Q  L  S  A  T  H  L  R  A  S  S  I  P  H  W  A  S  F  S  K

GACCCCTTCGCCTTTAGCAAG
 T  P  W  P  L  G  R
```

CODING SEGMENT L: (SEQ ID NO: 147)

```
  Y   V   S   A   N   T   T   P   A   R   K   S   P   V   D   P   H   T   P   S
  GTATGTATCAGCAATGACCACCCCGGCTCGTATGTCACCTGTAGATTTCCACACGCCAAG   60
  ||||||| |||||| |||||||||||||||||||||||||||||||||||||| |||||||
  gtatgtgtcagccatgaccacccggctcgtatgtcacctgtagatttccacacgccaag

  S   P   K   S   P   P   S   E   K   S   P   P   V   S   S   T   T   V   S   X
  CTCCCCCAAGTCACCCCGCTTCGGAAATGTCCCCGGCCCGTGTCCAGCACGACGGTCTCCAT  120
  ||||||||||  ||  ||||||||||||||||||| ||  |||||||||||| |||||| |||||
  ctcccccaaatcgcccccttcggaaatgtctccacccgtgtccagcatgacggtgtccat
                                                              N

  P   S   H   A   V   S   P   F   V   E   E   R   P   L   L   L   V   T   P
  GCCCTCCATGGCCGGTCAGTCCCTTCGTGGAAGAGGAGAGACCCCTGCTCCTTGTGACGCC  180
  ||| |||||||||||| |||||| |||||||| ||||||||| || || || |||||| ||
  gccttccatggcggtcagcccccttcatggaagaagagagagacctctacttctcgtgacacc
                |

  P   R   L   R   E   K   -   Y   D   H   H   A   Q   Q   F   N   S   F   E   C
  ACCACGGCTGCGGGAGAAG...TATGACCACCACGCCCAGCAATTCAACTCGTTCCACTG  240
  |||| |||||||||||||||   | |||| | ||| | ||||| || | ||| ||||||
  accaaggctgcgggagaagaagtttgaccatcaccctcagcagttcagctccttccacca
             K   F                   P           S           E

  N   P   A   K   E   S   N   S   L   P   P   S   P   L   R   I   V   E   D   S
  CAACCCCGCGCATGAGAGCAACAGCCTGCCCCCCAGCCCCTTGAGGATAGTGGAGGATGA  300
  |||||||||||||| || ||||||| || | |||||||||||||||||||||||||||||||||
  caaccccgcgcatgacagtaacagcctccctgctagcccttgaggatagtggaggatga
             D                   A

  E   Y   E   T   T   Q   E   Y   S   P   A   Q   E   P   V   K   K   L   P   I
  GGAATATGAAACGACCCAGGAGTACGAACCAGCTCAAGAGCCCGTTAAGAAACTCACCA  360
  |||| ||||||||||||||||||||| ||||||| ||||| ||||||| |||||||||||| ||||
  ggagtatgaaacgacccaagagtacgagccagcccaagagcctgttaagaaactcgcca
                                                              A

  S   S   R   R   A   K   R   T   K   P   N   G   H   I   A   N   R   L   E   K
  CAGCAGCCGGCGGGCCAAAAGAACCAAGCCCAATGGTCACATTGCCCACAGGTTGGAAT  420
  |||||||||||||||||||||||||||||||!!!!!!!!!! !!!!!!!!  |||| ||||!!! !
  ...tagccggcgggccaaaagaaccaagcccaatggccacattgctaacagattggaagt
                                                      N        V
```

FIGURE 31 (CONT.) (p. 8)

```
   D  N  N  T  G  A  D  S  S  N  S  E  S  E  T  E  D  E  R  V
GGACAACAACACAGGCGCTGACAGCAGTAACTCAGACAGCCAAACAGACGATGAAAGAGT    480
|||||  |||||||| ||| |  |  ||||||||||||||||||||  |||||||  ||||||||||||
ggacagcaacacaagctcccagagcagtaactcagagagtgaaacagaagatgaaagagt
        S        S  S  Q

   G  E  D  T  P  P  L  A  I  Q  N  P  L  A  A  S  L  E  A  A
AGGAGAAGATACGCCTTcCTGGCCATACAGAACCCCcTGGCAGCCAGTCTCGAGGCCGGC    540
|||  ||||||||||||||||||||  ||||||||||||||||||||||||||  |||||  |
aggtgaagatacgcctttcctgggcatacagaacccccctggcagccagtcttgaggcaac
        G                                                   T

   P  A  F  R  L  V  D  S  R  T  N  P  T  G  G  F  S  P  Q  E
CCCTGCCTTCCGCCTGGTCGACAGCAGGACTAACCCAACAGGCCGGCTTCTCTCCGCAGGA    600
||||||||||||||||  ||||||||||||||||  ||||||  |||||||  |  |||||||
acctgccttccgcctggctgacagcaggactaacccagcaggccgcttctcgacacagga
        A                            A  E              T

   E  L  Q  A  R  L  S  G  V  I  A  N  Q  D  P  I  A  V  *
AGAATTGCAGGCCAGGCTCTCCGGTGTAATCGCTAACCAAGACCCTATCGCTGTCTAAA    660
|||||  |  ||||||||||||  ||  ||||||||  |||||||||||||||||||  |||||  |||||
agaaatccaggccaggctgtctagtgtaattgctaaccaagaccctattgctgtataaa
        I                        S

CCGAAATACACCCATAGATTCACCTGTAAAACTTTATTTTATATAATAAGTATTCCACC    720
||  |||||| ||  |||||||||||||||||||||||||||||||||||||||||||||||||||||||
cctaaataaacacatagattcacctgtaaaactttattttatataataaagtattccacc

TTAAATTAAACAA    733
|||||||||||||
ttaaattaaacaa
```

FIGURE 31 (CONT.) (8 of 8)

Human Coding Segment E: (SEQ ID NO: 163)

```
ATGAGATGGCCGACGCGCCCcgCgCcGCTCCGGGCCGTCCCGGCCCCCGGGCCCAGCGCCCC   60
 M  R  W  R  R  A  P  R  R  S  G  R  P  G  P  R  A  Q  R  P

GGCTCCGCCGCCGCTCGTCGCCGCCGCTGCCGCTGCTGCCACTACTGCTGCTGCTGGGG   120
 G  S  A  A  R  S  S  P  P  L  P  L  L  P  L  L  L  L  G

ACCGCGGCCCTGGCGCCGGGGCGGCGGCCGGCAACGAGGCGGCTCCCGCGGGGGCCTCG   180
 T  A  A  L  A  P  G  A  A  A  G  N  E  A  A  P  A  G  A  S

GTGTGCTACTCGTCCCCGCCCAGCGTGGGATCGGTGCAGGAGCTAGCTCAGCGCGCCGCG   240
 V  C  Y  S  S  P  P  S  V  G  S  V  Q  E  L  A  Q  R  A  A

GTGGTGATCGAGGGAAAGGTGCACCCGCAGCGGCGGCAGCAGGGGGCACTCGACAGGAAG   300
 V  V  I  E  G  K  V  H  P  Q  R  R  Q  Q  G  A  L  D  R  K

GCGGCGGCGGCGGGGCGAGGCAGGGGCGTGGGCGGCGATCGCGAGCCGCCAGCCGCG   360
 A  A  A  A  A  G  E  A  G  A  W  G  G  D  R  E  P  P  A  A

GGCCCACGGGCGCTGGGGCCGCCCGCCGAGGAGCCGGCTGCTCGCCGCCAACGGGACCGTG   420
 G  P  R  A  L  G  P  P  A  E  E  P  L  L  A  A  N  G  T  V

CCCTCTTGGCCCACCGCCCCGGTGCCCAGCGCCGGCGAGCCCGGGGAGGAGCCGCCCTAT   480
 P  S  W  P  T  A  P  V  P  S  A  G  E  P  G  E  E  A  P  Y

CTGGTGAAGGTGCACCAGGTGTGGGCGGTGAAAGCCGGGGGCTTGAAGAAGGACTCGCTG   540
 L  V  K  V  H  Q  V  W  A  V  K  A  G  G  L  K  K  D  S  L

CTCACCGTGCGCCTGGGGACCTGGGGCCACCCCGCCTTCCCCTCCTGCGGGAGGCTCAAG   600
 L  T  V  R  L  G  T  W  G  H  P  A  F  P  S  C  G  R  L  K

GAGGACAGCAGGTACATCTTCTTCATGGAGCCCGACGCCAACAGCACCAGCCGCGCGCCG   660
 E  D  S  R  Y  I  F  F  M  E  P  D  A  N  S  T  S  R  A  P

GCCGCCTTCCGAGCCTCTTTCCCCCCTCTGGAGACGGGCCGGAACCTCAAGAAGGAGGTC   720
 A  A  F  R  A  S  F  P  P  L  E  T  G  R  N  L  K  K  E  V

AGCCGGGTGCTGTGCAAGCGGTGCG   749
 S  R  V  L  C  K  R  C
```

HUMAN GGF CODING SEGMENT A'

GAAGTCAGAACTTCGCATTAACAAAGCATCACTCGCTGATTCTCGAGAGTATATGTGCAA 60
 K S E L R I N K A S L A D S G E Y M C X

AGTGATCAGCAAATTAGGAAATCACAGTGCCTCTCCAATATCACCATCGTCGAATCAAA 120
 V I S K L G N D S A S A N I T I V E S N

CGGTAAGAGATACCTACGGTATTCTGTTCCTCAATCTGTAACAAGAGTAATCAAAACATG 180
 G K R Y L R Y S V P Q S V T R V I K T C

TGGTAAGACTCATAATAGACTGGTGTGTTAA   211
 G K T H N R L V C *

(SEQ ID NO: 168)

(SEQ ID NO: 148) (1 - 2)   FIGURE 32

GGF2BPP5 nucleotide sequence and deduced protein sequence

AGTTTCCCCCCCCAACTTGTCCGAACTCTGGGCTCGCGCGCAGGGCAGGAGCGGAGCCGC 60

GGCGGCTGCCCAGGCGATGCCGAGCGCCGGCCCGACCGTAATCGCCTCTCCGTCCTCGGC 120

TGCGAGCGCCGCCGGACCGAGGCAGCGACAGGAGCGGACCGCGGCCGGGAACCGAGGACTCC 180

CCAGCCGCGCCGCCAGCAGGAGCCACCCCGCGAGTCGTGCGACCCGGGACGGAGCGCCCGCC 240

AGTCCCAGGTGGCCCGGACCGCACGTTCGTCCCCGCGCTCCCCGCCGGCGACAGGAGAC 300

GCTCCCCCCCACGCCGCGCGCGCCTCGGCCCGGTCGCTGGCCCGCCTCCACTCCGGGGAC 360

AAACTTTTCCCGAAGCCGATCCCAGCCCTCGGACCCAAACTTGTCGCGCGTCGCCTTCGC 420

CGGGAGCCGTCCGCGCAGAGCCGTGCCACTTCTCGGGCGAGATGTCGGAGCGCAGAGAAGGC 480
                                        M S E R R E G

AAAGGCAAGGGGAAGGGCCGGCAAGAAGGACCGAGGCTCCGGGAAGAAGCCCGTGCCCGCG 540
K G K G K G G K K D R G S G K K P V P A

GCTGGCGGCCCCGAGCCCAGCCTTGCCTCCCCGCTTGAAAGAGATGAAGAGTCAGGAGTCT 600
A G G P S P A L P P R L K E M K S Q E S

GTGGCAGGTTCCAAACTAGTGCTTCGGTGCGAGACCAGTTCTGAATACTCCTCTCTCAAG 660
V A G S K L V L R C E T S S E Y S S L K

TTCAAGTGGTTCAAGAATGGCAGTGAATTAAGCCGAAAGAACAAACCACAAACATCAAG 720
F K W F K N G S E L S R K N K P Q N I K

ATACAGAAAAGGCCGGGGAAGTCAGAACTTCGCATTAGCAAAGCGTCACTGGCTGATTCT 780
I Q K R P G K S E L R I S K A S L A D S

GGAGAATATATGTGCAAAGTGATCAGCAAACTAGGAAATGACAGTGCCTCTGCCAACATC 840
G E Y M C K V I S K L G N D S A S A N I

ACCATTGTGGAGTCAAACGAGATCACCACTGGCATGCCAGCCTCAACTGAGACAGCCTAT 900
T I V E S N E I T T G M P A S T E T A Y

GTGTCTTCAGAGTCTCCCATTAGAATATCAGTATCAACAGAAGGAACAAATACTTCTTCA 960
V S S E S P I R I S V S T E G T N T S S

TCCACATCCACATCTACAGCTGGGACAAGCCATCTTGTCAAGTGTGCAGAGAAGGAGAAA 1020
S T S T S T A G T S H L V K C A E K E

ACTTTCTGTGTGAATGGAGGCCAGTGCTTCATGGTGAAAGACCTTTCAAATCCCTCAAGA 1080
T F C V N G G E C F M V K D L S N P S R

TACTTGTGCAAGTGCCCAAATGAGTTTACTGGTGATCGCTGCCAAAACTACGTAATGGCC 1140
Y L C K C P N E F T G D R C Q N Y V M A

AGCTTCTACAGTACGTCCACTCCCTTTCTGTCTCTGCCTGAATAGGCCATGCTCAGTCC 1200
S F Y S T S T P F L S L P E *

GTGCCCGTGCCCCGCCATCCCCATCACACCAATCCACATCTACAGCCTAGATGCCTTTA 1260

............................ACACATCCCATTCCA ....

FIGURE 32 (CONT.) (2 of _)

TGACTTCCTCTCTCCGTCACTAGTGGGCTCTCAGCTACTCGTAGGTCCGTAAGGCTCCAG 1380

TGTTTCTGAAATTGATCTTCAATTACTCTGATACGACATGATAGTCCCTCTCACCCAGTC 1440

CAATCACAATAAAGGCCTTCAAAGTCTCACTTTTATTCAGAAAATAAAAATCGTTCCAC 1500

GGGACAGTCCCTCTTCTTTATAAAATGACCCTATCCTTGAAAGGAGGTGTGTTAAGTTC 1560

TAACCAGTACACACTTCAAATCATGGTAAGTTCGCTTCGGTTCAGAATGTGTTCTTTCTC 1620

ACAAATAAACAGAATAAAAAAAAAAAAAAAA 1653

(SEQ ID NO: 149)   FIGURE 33

GGF2BPP2 nucleotide sequence and deduced protein sequence

```
CATCATGTGTGGgCGGCgAAAGCCgCGGGGCTTGAAGAAgGACTCGCTGCTCACCGTgCgc   60
M  Q  V  W  A  A  K  A  G  G  L  K  K  D  S  L  L  T  V  R

CTGGGCgCCTGGgGCCACCCCGCCTTCCCCTCCTgCGGCCGCCTCAAGGAGGACAGCAGG   120
L  G  A  W  G  H  P  A  F  P  S  C  G  R  L  K  E  D  S  R

TACATCTTCTTCATGGAgCCCGAGGCCAACAGCAgcggCgGgCCCGGCCgCCTTCCGAGC   180
Y  I  F  F  M  E  P  E  A  N  S  S  G  G  P  G  R  L  P  S

CTCCTTCCCCCCTCTCGAGACGGGCCGGAACCTCAAGAAGGAGgTCAGCCGGGTGCTGT   240
L  L  P  P  S  R  D  G  P  E  P  Q  E  G  G  Q  P  G  A  V

CAACgGTGCGGCCTTGCCTCCCCGGCTTGAAgGAGATGAAGAGTCAGGAGTCTGTGGCAGGT   300
Q  R  C  A  L  P  P  R  L  K  E  M  K  S  Q  E  S  V  A  G

TCCAAACTAGTGCTTCGGTGCGAGACCAGTTCTGAATACTCCTCTCTCAAGTTCAAgTGG   360
S  K  L  V  L  R  C  E  T  S  S  E  Y  S  S  L  K  F  K  W

tTCAAgAATGGGAGTCaaTTAAGCCGAAAGAACAAACCAgAAAACATCAAGATACAGAAA   420
F  K  N  G  S  E  L  S  R  K  N  K  P  E  N  I  K  I  Q  K

AGGCCCGGGAAGTCAGAACTTCGCATTAGCAAAGCGTCACTGGCTGATTCTGGAGAATAT   480
R  P  G  K  S  E  L  R  I  S  K  A  S  L  A  D  S  G  E  Y

ATGTGCAAAGTGATCAGCAAACTAGGAAATGACAGTGCCTCTGCCAACATCACCATTGTG   540
M  C  K  V  I  S  K  L  G  N  D  S  A  S  A  N  I  T  I  V

GAGTCAAACGCCACATCCACATCTACAGCTGGGACAAGCCATCTTGTCAAGTGTGCAGAG   600
E  S  N  A  T  S  T  S  T  A  G  T  S  L  V  K  C  A  E

AAGGAGAAAACTTTCTGTGTGAATGGAGGCGAGTGCTTCATGGTGAAAGACCTTTCAAAT   660
K  E  K  T  F  C  V  N  G  G  E  C  F  M  V  K  D  L  S  N

CCCTCAAGATACTTGTGCAAGTGCCAACCTGGATTCACTGGAGCGAGATGTACTGAGAAT   720
P  S  R  Y  L  C  K  C  Q  P  G  F  T  G  A  R  C  T  E  N

GTGCCCATGAAAGTCCAAACCCAAGAAAGTGCCCAATGAGTTTACTGGTGATCGCTGC   780
V  P  M  K  V  Q  T  Q  E  K  C  P  N  E  F  T  G  D  R  C

CAAAACTACGTAATGGCCAGCTTCTACAGTACGTCCACTCCCTTTCTGTCTCTGCCTGAA   840
Q  N  Y  V  M  A  S  F  Y  S  T  S  T  P  F  L  S  L  P  E

TAGCGGCATCTCAGTCGGTGCCGGCTTTCTTGTTGCCGCATCTCCCCTCAGATTCCTGCTAG   900
*

AGCTAGATGCGTTTTACCAGGTCTAACATTGACTGCCTCTGCCTGTCGCATGAGAACATT   960

AACACAAGCCGATTGTATGACTTCCTCTGTCCGTGACTAGTCGGCTCTGAGCTACTCGTAG   1020

GTCCGTAAGGCTCCAGTGTTTCTGAAATTGATCTTGAATTACTGTCATaCGACATCATAC   1080

TCCCTCTCACCCAGTGCCAATGACAATAAAGGCCTTCAAAAGTCAAAAAAAAAAAAAAAA   1140
```

(SEQ ID NO: 150) (1 of 2)   FIGURE 34

GGF2BPP4 nucleotide sequence and deduced protein sequence

```
GAAGTCAGAACTTCGCATTAGCAAAGCCGTCACTGGCTGATTCTGGAGAACATATGTGCAA   60
 K  S  E  L  R  I  S  K  A  S  L  A  D  S  G  E  Y  M  C  K

AGTGATCAGCAAACTAGGAAATGACAGTGCCTCTGCCAACATCACCATTGTGGAGTCAAA  120
 V  I  S  K  L  G  N  D  S  A  S  A  N  I  T  I  V  E  S  K

CGCCACATCCACATCTACAGCTGGGACAAGCCATCTTGTCAAGTGTGCAGAGAAGGAGAA  180
 A  T  S  T  S  T  A  G  T  S  H  L  V  K  C  A  E  K  E  K

AACTTTCTGTGTGAATGGAGGCGACTGCTTCATGGTGAAAGACCTTTCAAATCCCTCAAG  240
 T  F  C  V  N  G  G  D  C  F  M  V  K  D  L  S  N  P  S  R

ATACTTGTGCAAGTGCCAACCTGGATTCACTGGAGCCAGATGTACTGAGAATGTGCCCAT  300
 Y  L  C  K  C  Q  P  G  F  T  G  A  R  C  T  E  N  V  P  M

GAAAGTCCAAACCCAAGAAAAGCGGAGGAGCTCTACCAGAAGAGAGTGCTCACCATTAC  360
 K  V  Q  T  Q  E  K  A  E  E  L  Y  Q  K  R  V  L  T  I  T

CGGCATTTGCATCGCGCTGCTCGTGGTTGGCATCATGTGTGTGGTGGTCTACTGCAAAC  420
 G  I  C  I  A  L  L  V  V  G  I  M  C  V  V  V  Y  C  K  T

CAAGAAACAACGGAAAAGCTTCATGACCGGCTTCGGCAGAGCCTTCGGTCTGAAAGAA  480
 K  K  Q  R  K  K  L  H  D  R  L  R  Q  S  L  R  S  E  R  N

CACCATGATGAACGTAGCCAACGGCCCCACCACCCCAATCCGCCCCCCGAGAACGTGCA  540
 T  M  M  N  V  A  N  G  P  H  H  P  N  P  P  P  E  N  V  Q

GCTGGTGAATCAATACGTATCTAAAAATGTCATCTCTAGCGAGCATATTGTTGAGAGAGA  600
 L  V  N  Q  Y  V  S  K  N  V  I  S  S  E  H  I  V  E  R  E

GGCGGAGAGCTCTTTTTCCACCAGTCACTACACTTCGACAGCTCATCATTCCACTACTGT  660
 A  E  S  S  F  S  T  S  H  Y  T  S  T  A  H  H  S  T  T  V

CACTCAGACTCCCAGTCACAGCTGGAGCAATGGACACACTGAAAGCATCATTTCGGAAG  720
 T  Q  T  P  S  H  S  W  S  N  G  H  T  E  S  I  I  S  E  S

CCACTCTGTCATCGTGATGTCATCCGTAGAAAACAGTAGGCACAGCAGCCCGACTGGGG  780
 H  S  V  I  V  M  S  S  V  E  N  S  R  H  S  S  P  T  G  G

CCCGAGAGGACGTCTCAATGGCTTGGGAGGCCCTCGTGAATGTAACAGCTTCCTCAGGCA  840
 P  R  G  R  L  N  G  L  G  G  P  R  E  C  N  S  F  L  R  H

TGCCAGAGAAACCCCTGACTCCTACCGAGACTCTCCTCATAGTGAAAGACATAACCTTAT  900
 A  R  E  T  P  D  S  Y  R  D  S  P  H  S  E  R  H  N  L  I

AGCTGAGCTAAGGAGAAACAAGGCCCACAGATCCAATGCATGCAGATCCAGCTTTCCGC  960
 A  E  L  R  R  H  K  A  H  R  S  K  C  H  Q  I  Q  L  S  A

AACTCATCTTAGAGCTTCTTCCATTCCCCATTGGGCTTCATTCTCTAAGACCCCTTGGCC  1020
 T  H  L  R  A  S  S  I  P  H  W  A  S  F  S  K  T  P  W  P

TTCAGCAAGGTATGTATCAGCAATGACCACCCCGGCTCGTATGTCACCTGTAGATTTCCA  1080
 L  G  R  Y  V  S  A  H  T  T  P  A  R  H  S  P  V  D  F  H
```

· ·

FIGURE 34 (CONT.) (2 of _)

```
GGTCTCCATGCCCTCCATGGCCGTCAGTCCCTTCGTGGAAGAGGAGAGACCCCTGCTCCT   1200
 V  S  M  P  S  M  A  V  S  P  F  V  E  E  E  R  P  L  L  L

TGTGACGCCACCACGGCTGCGGGAGAAGTATGACCACCACGCCCAGCAATTCAACTCGTT   1260
 V  T  P  P  R  L  R  E  K  Y  D  H  H  A  Q  Q  F  N  S  F

CCACTGCAACCCCGCGCATGAGAGCAACAGCCTGCCCCCCAGCCCCTTGAGGATAGTGGA   1320
 H  C  N  P  A  H  E  S  N  S  L  P  P  S  P  L  R  I  V  E

GGATGAGGAATATGAAACGACCCAGGAGTACGAACCAGCTCAAGAGCCGGTTAAGAAACT   1380
 D  E  E  Y  E  T  T  Q  E  Y  E  P  A  Q  E  P  V  K  K  L

CACCAACAGCAGCCGGCGGGCCAAAAGAACCAAGCCCAATGGTCACATTGCCCACAGGTT   1440
 T  N  S  S  R  R  A  K  R  T  K  P  N  G  H  I  A  H  R  L

GGAAATGGACAACAACACAGGCGCTGACAGCAGTAACTCAGAGAGCGAAACAGAGGATGA   1500
 E  M  D  N  N  T  G  A  D  S  S  N  S  E  S  E  T  E  D  E

AAGAGTAGGAGAAGATACGCCTTTCCTGGCCATACAGAACCCCGTGGCAGCCAGTCTCGA   1560
 R  V  G  E  D  T  P  F  L  A  I  Q  N  P  L  A  A  S  L  E

GGCGGCCCCTGCCTTCCGCCTGGTCGACAGCAGGACTAACCCAACAGGCGGCTTCTCTCC   1620
 A  A  P  A  F  R  L  V  D  S  R  T  N  P  T  G  G  F  S  P

GCAGGAAGAATTGCAGGCCAGGCTCTCCGGTGTAATCGCTAACCAAGACCCTATCGCTGT   1680
 Q  E  E  L  Q  A  R  L  S  G  V  I  A  N  Q  D  P  I  A  V

CTAAAACCGAAATACACCCATAGATTCACCTGTAAAACTTTATTTTATATAATAAAGTAT   1740
 *

TCCACCTTAAATTAAACAAAAAAA   1764
```

FIGURE 35

```
           •        •        •              • •            •
GGF2boo5KCAEKEKTFCVNGGECFMVKDLSNPSRYLCKCPNEFTGDRCQNYVMASFY 1
GGF2boo4KCAEKEKTFCVNGGDCFMVKDLSNPSRYLCKCQPGFTGARCTENVPMKVQ 2
hEGE     ECLRKYKDFCIH - GECKYVKELRAPS — CKCQQEYFGERCGEKSNKTHS 3
       •
```

[1](SEQ ID NO: 151)
[2](SEQ ID NO: 152)
[3](SEQ ID NO: 153)

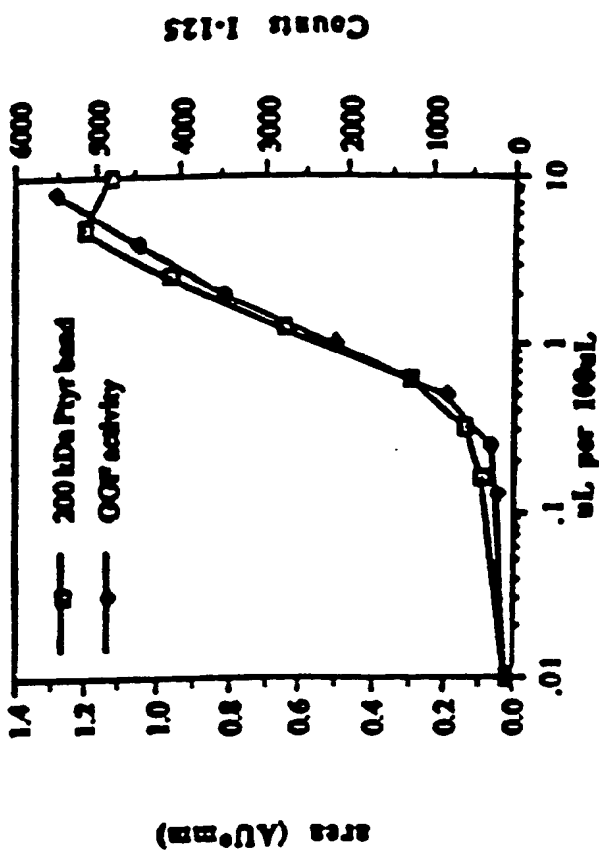200 kDa tyrosine phosphorylation compared with mitogenic activity

FIGURE 37 (1 of 2)

GGF/HEREGULIN SPLICING VARIANTS

F-B-A'

F-B-A-C-C/D-D
F-B-A-C-C/D-H
F-B-A-C-C/D-H-L
F-B-A-C-C/D-H-K-L
F-B-A-C-C/D-D'-H
F-B-A-C-C/D-D'-H-L
F-B-A-C-C/D-D'-H-K-L
F-B-A-C-C/D'-D
F-B-A-C-C/D'-H
F-B-A-C-C/D'-H-L
F-B-A-C-C/D'-H-K-L
F-B-A-C-C/D'-D'-H
F-B-A-C-C/D'-D'-H-L
F-B-A-C-C/D'-D'-H-K-L
F-B-A-C-C/D-C/D'-D
F-B-A-C-C/D-C/D'-H
F-B-A-C-C/D-C/D'-H-L
F-B-A-C-C/D-C/D'-H-K-L
F-B-A-C-C/D-C/D'-D'-H
F-B-A-C-C/D-C/D'-D'-H-L
F-B-A-C-C/D-C/D'-D'-H-K-L

F-E-B-A'

F-E-B-A-C-C/D-D
F-E-B-A-C-C/D-H
F-E-B-A-C-C/D-H-L
F-E-B-A-C-C/D-H-K-L
F-E-B-A-C-C/D-D'-H
F-E-B-A-C-C/D-D'-H-L
F-E-B-A-C-C/D-D'-H-K-L
F-E-B-A-C-C/D'-D
F-E-B-A-C-C/D'-H
F-E-B-A-C-C/D'-H-L
F-E-B-A-C-C/D'-H-K-L
F-E-B-A-C-C/D'-D'-H
F-E-B-A-C-C/D'-D'-H-L
F-E-B-A-C-C/D'-D'-H-K-L
F-E-B-A-C-C/D-C/D'-D
F-E-B-A-C-C/D-C/D'-H
F-E-B-A-C-C/D-C/D'-H-L
F-E-B-A-C-C/D-C/D'-H-K-L
F-E-B-A-C-C/D-C/D'-D'-H
F-E-B-A-C-C/D-C/D'-D'-H-L
F-E-B-A-C-C/D-C/D'-D'-H-K-L

F-B-A-G-C-C/D-D
F-B-A-G-C-C/D-H
F-B-A-G-C-C/D-H-L
F-B-A-G-C-C/D-H-K-L
F-B-A-G-C-C/D-D'-H
F-B-A-G-C-C/D-D'-H-L
F-B-A-G-C-C/D-D'-H-K-L
F-B-A-G-C-C/D'-D
F-B-A-G-C-C/D'-H
F-B-A-G-C-C/D'-H-L
F-B-A-G-C-C/D'-H-K-L
F-B-A-G-C-C/D'-D'-H
F-B-A-G-C-C/D'-D'-H-L
F-B-A-G-C-C/D'-D'-H-K-L
F-B-A-G-C-C/D-C/D'-D
F-B-A-G-C-C/D-C/D'-H
F-B-A-G-C-C/D-C/D'-H-L
F-B-A-G-C-C/D-C/D'-H-K-L
F-B-A-G-C-C/D-C/D'-D'-H
F-B-A-G-C-C/D-C/D'-D'-H-L
F-B-A-G-C-C/D-C/D'-D'-H-K-L

F-E-B-A-G-C-C/D-D
F-E-B-A-G-C-C/D-H
F-E-B-A-G-C-C/D-H-L
F-E-B-A-G-C-C/D-H-K-L
F-E-B-A-G-C-C/D-D'-H
F-E-B-A-G-C-C/D-D'-H-L
F-E-B-A-G-C-C/D-D'-H-K-L
F-E-B-A-G-C-C/D'-D
F-E-B-A-G-C-C/D'-H
F-E-B-A-G-C-C/D'-H-L
F-E-B-A-G-C-C/D'-H-K-L
F-E-B-A-G-C-C/D'-D'-H
F-E-B-A-G-C-C/D'-D'-H-L
F-E-B-A-G-C-C/D'-D'-H-K-L
F-E-B-A-G-C-C/D-C/D'-D
F-E-B-A-G-C-C/D-C/D'-H
F-E-B-A-G-C-C/D-C/D'-H-L
F-E-B-A-G-C-C/D-C/D'-H-K-L
F-E-B-A-G-C-C/D-C/D'-D'-H
F-E-B-A-G-C-C/D-C/D'-D'-H-L
F-E-B-A-G-C-C/D-C/D'-D'-H-K-L

FIGURE 37 (CONT.)   (2 of 2)

OGF/HEREGULIN SPLICING VARIANTS CONTINUED

E-B-A'

E-B-A-C-C/D-D
E-B-A-C-C/D-H
E-B-A-C-C/D-H-L
E-B-A-C-C/D-H-K-L
E-B-A-C-C/D-D'-H
E-B-A-C-C/D-D'-H-L
E-B-A-C-C/D-D'-H-K-L
E-B-A-C-C/D'-D
E-B-A-C-C/D'-H
E-B-A-C-C/D'-H-L
E-B-A-C-C/D'-H-K-L
E-B-A-C-C/D'-D'-H
E-B-A-C-C/D'-D'-H-L
E-B-A-C-C/D'-D'-H-K-L
E-B-A-C-C/D-C/D'-D
E-B-A-C-C/D-C/D'-H
E-B-A-C-C/D-C/D'-H-L
E-B-A-C-C/D-C/D'-H-K-L
E-B-A-C-C/D-C/D'-D'-H
E-B-A-C-C/D-C/D'-D'-H-L
E-B-A-C-C/D-C/D'-D'-H-K-L

E-B-A-G-C-C/D-D
E-B-A-G-C-C/D-H
E-B-A-G-C-C/D-H-L
E-B-A-G-C-C/D-H-K-L
E-B-A-G-C-C/D-D'-H
E-B-A-G-C-C/D-D'-H-L
E-B-A-G-C-C/D-D'-H-K-L
E-B-A-G-C-C/D'-D
E-B-A-G-C-C/D'-H
E-B-A-G-C-C/D'-H-L
E-B-A-G-C-C/D'-H-K-L
E-B-A-G-C-C/D'-D'-H
E-B-A-G-C-C/D'-D'-H-L
E-B-A-G-C-C/D'-D'-H-K-L
E-B-A-G-C-C/D-C/D'-D
E-B-A-G-C-C/D-C/D'-H
E-B-A-G-C-C/D-C/D'-H-L
E-B-A-G-C-C/D-C/D'-H-K-L
E-B-A-G-C-C/D-C/D'-D'-H
E-B-A-G-C-C/D-C/D'-D'-H-L
E-B-A-G-C-C/D-C/D'-D'-H-K-L

FIGURE 38
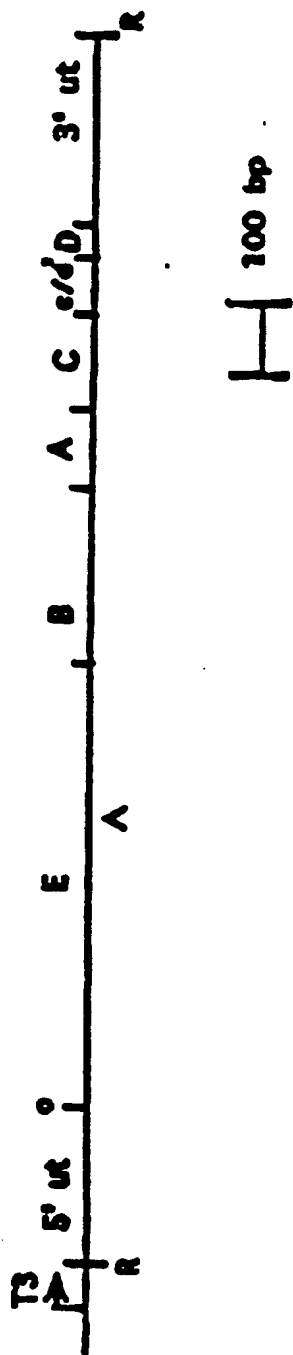
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AGCCATCTTGTCAAGTGTGCAGAGAAGGAGAAAACTTTCTGTGTGAATGGAGGCGAGTGC
S   H   L   V   K   C   A   E   K   E   K   T   F   C   V   N   G   G   E   C

TTCATGGTGAAAGACCTTTCAAATCCCTCAAGATACTTGTGCAAGTGCCCAAATGAGTTT
F   M   V   K   D   L   S   N   P   S   R   Y   L   C   K   C   P   N   E   F

ACTGGTGATCGCTGCCAAAACTACGTAATGGCCAGCTTCTACAGTACGTCCACTCCCTTT
T   G   D   R   C   Q   N   Y   V   M   A   S   F   Y   S   T   S   T   P   F

CTGTCTCTGCCTGAATAG
L   S   L   P   E   *

FIGURE 39

(SEQ ID NO: 154)

EGFL2

```
AGCCATCTTGTCAAGTGTGCAGAGAAGGAGAAAACTTTCTGTGTGAATGGAGGCGAGTGC
S   H   L   V   K   C   A   E   K   E   K   T   F   C   V   N   G   G   E   C

TTCATGGTGAAAGACCTTTCAAATCCCTCAAGATACTTGTGCAAGTGCCAACCTGGATTC
F   M   V   K   D   L   S   N   P   S   R   Y   L   C   K   C   Q   P   G   F

ACTGGAGCGAGATGTACTGAGAATGTGCCCATGAAAGTCCAAACCCAAGAAAAGCGGAG
T   G   A   R   C   T   E   N   V   P   M   K   V   Q   T   Q   E   K   A   E

GAGCTCTACTAA
E   L   Y   *
```

FIGURE 40

(SEQ ID NO: 155)

IGFL?

```
AGCCATCTTGTCAAGTGTGCAGAGAAGGAGAAAACTTTCTGTGTGAATGGAGGCCAGTGC
 S  H  L  V  K  C  A  E  K  E  K  T  F  C  V  N  G  G  E  C

TTCATGGTGAAAGACCTTTCAAATCCCTCAAGATACTTGTGCAAGTGCCCAAATGAGTTT
 F  M  V  K  D  L  S  N  P  S  R  Y  L  C  K  C  P  N  E  F

ACTGGTGATCGCTGCCAAAACTACGTAATGGCCAGCTTCTACAAAGCGGAGGAGCTCTAC
 T  G  D  R  C  Q  N  Y  V  M  A  S  F  Y  K  A  E  E  L  Y

TAA
```

FIGURE 41

(SEQ ID NO: 156)

EGFL4

```
AGCCATCTTGTCAAGTGTGCAGAGAAGGAGAAAACTTTCTGTGTGAATGGAGGCGAGTGC
 S  H  L  V  K  C  A  E  K  E  K  T  F  C  V  N  G  G  E  C

TTCATGGTGAAAGACCTTTCAAATCCCTCAAGATACTTGTGCAAGTGCCCAAATGAGTTT
 F  M  V  K  D  L  S  N  P  S  R  Y  L  C  K  C  P  N  E  F

ACTGGTGATCGCTGCCAAAACTACGTAATGGCCAGCTTCTACAAGCATCTTGGGATTGAA
 T  G  D  R  C  Q  N  Y  V  M  A  S  F  Y  K  H  L  G  I  E

TTTATGGAGAAAGCGGAGGAGCTCTACTAA
 F  M  E  K  A  E  E  L  Y  *
```

FIGURE 42

(SEQ ID NO: 157)

EGFL5

```
AGCCATCTTGTCAAGTGTGCAGAGAAGGAGAAAACTTTCTGTCTGAATGGAGGCGAGTGC
 S  H  L  V  K  C  A  E  K  E  K  T  F  C  V  N  G  G  E  C

TTCATGGTGAAAGACCTTTCAAATCCCTCAAGATACTTGTGCAAGTGCCAACCTGGATTC
 F  M  V  K  D  L  S  N  P  S  R  Y  L  C  K  C  Q  P  G  F

ACTGGAGCGAGATGTACTGAGAATGTGCCCATGAAAGTCCAAACCCAAGAAAGTGCCCA
 T  G  A  R  C  T  E  N  V  P  M  K  V  Q  T  Q  E  K  C  P

AATGAGTTTACTGGTGATCGCTGCCAAAACTACGTAATGGCCAGCTTCTACAGTACGTCC
 N  E  F  T  G  D  R  C  Q  N  Y  V  M  A  S  F  Y  S  T  S

ACTCCCTTTCTGTCTCTGCCTGAATAG
 T  P  F  L  S  L  P  E  *
```

FIGURE 43

(SEQ ID NO: 158)

EGFL4

```
AGCCATCTTGTCAAGTGTGCAGAGAAGGAGAAAACTTTCTGTGTGAATGGAGGCGAGTGC
 S  H  L  V  K  C  A  E  K  E  K  T  F  C  V  N  G  G  E  C

TTCATGGTGAAAGACCTTTCAAATCCCTCAAGATACTTGTGCAAGTGCCAACCTGGATTC
 F  M  V  K  D  L  S  N  P  S  R  Y  L  C  K  C  Q  P  G  F

ACTGGAGCGAGATGTACTGAGAATGTGCCCATGAAAGTCCAAACCCAAGAAAGTGCCCA
 T  G  A  R  C  T  E  N  V  P  M  K  V  Q  T  Q  E  K  C  P

AATGAGTTTACTGGTGATCGCTGCCAAAACTACGTAATGGCCAGCTTCTACAAAGCGGAG
 N  E  F  T  G  D  R  C  Q  N  Y  V  M  A  S  F  Y  K  A  E

GAGCTCTACTAA
 E  L  Y  *
```

FIGURE 44

(SEQ ID NO: 159)

Nucleotide sequence and deduced amino acid sequence of OGP2HBS5

```
GCAATTCCTTTTTTTTTTTTTTTTTTTTCttCtaaTTTTTTTTTTGCCCTTATACCTCTTCCCC    60
TTTCTGTGGTTCCATCCACTTCTTCCCCCTCCTCCTCCCATAAACAACTCTCCTACCCCT       120
GCACCCCCAATAAATAAATAAAACCAGGAGGGCCAAGGGGCGAGCAGCACGAGTGGTGCTG       180
CCGAGGGCAAGGAAAAGGGGAGGCCACGCGCAGAGAGGCCGGGCAGAGTCGGAACCGACAGCC    240
AGAAGCCGGGCACGCACCTCGCCACCATGAGGATGGGCTACGCGCCCGCGCGCCGCTCCGGGCGT  300
                              M  R  W  R  R  A  P  R  R  S  G  R
CCCGGCCCCGGGGCCCAGGGCCCCGGGCTCGGCCCGCGGCTCGTGCCCCGCGCTGCCGCTG       360
P  G  P  R  A  Q  R  P  G  S  A  A  R  S  S  P  P  L  P  L
CTGCCACTACTGCTGCTGCTGGGCACCGCCGGCCCTGGCCCCGGGGCGGCGGCCGGCAAC       420
L  P  L  L  L  L  L  G  T  A  A  L  A  P  G  A  A  A  G  N
      V  C  L  L  T  V  A  A  L  P  P  T
                    OGPII-9
CAGCCGGCTCCCGCGGGCGCCTCGGTGTGCTACTCGTCCCGCGCCCACCGTGGGATCGGTG      480
E  A  A  P  A  G  A  S  V  C  Y  S  S  P  P  S  V  G  S  V
                            A  S  P  V  S  V  G  S  V
                                  OGPII-8
CAGCAGCTGCTCAGCGCGCCGCGCGGTCGTCATCGAGGGAAAGGTCCACCGGCAGCCGCGG      540
Q  E  L  A  Q  R  A  A  V  V  I  E  G  K  V  E  P  Q  R  R
Q  E  L  V  Q  R  W  P  V  Y  I  E  G  K
          |           OGPII-4
CAGCAGGGGGCACTCCACAGGAAGCCGGCGGCGGCGGCGGCGGGCGAGGGCGATGGGC      600
Q  Q  G  A  L  D  R  K  A  A  A  A  A  G  E  A  G  A  W  G
GGGCATCGCGAGCCGCCGGCGGCCGGGCCCAGGGCGCTGGGCCCGCCGGCGGAGGAGCCG    660
G  D  R  E  P  P  A  A  G  P  R  A  L  G  P  P  A  E  E  P
CTGCTGGCGGCCAACGGCACGGTGCCCTCTTGGGCCCACCGCCCCGGTGCCCAGCGCCGGC    720
L  L  A  A  N  G  T  V  P  S  W  P  T  A  P  V  P  S  A  G
```

FIGURE 45 (1 of 3)

(SEQ ID NO: 167)

FIGURE 45 (2 of 3)

(SEQ ID NO: 167)

TCCACCACTGGGACAAGCCATCTTGTAAAATGTGCGGCAGAAGGAGAAAACTTTCTGTGTG 1380
S T T G T S S L V K C A E K K K T F C V

AATCGAGGGGAGTGCTTCATGGTGAAAGACCTTTCAAACCCCTGGAGATACTTGTGCAAG 1440
N G G E C F M V K D L S N F S R Y L C K

TGCCCAAATGAGTTTACTGGTGATCGCTGCCAAAACTACGTAATGGCCAGCTTCTACAGT 1500
C P N E F T G D R C Q N Y V M A S F Y S

ACGTCGACTCCGTTTCTGTCTCTGCCTGAATAGGAGCATGCTCAGTTCGTGCTGCTTTCT 1560
T S T F F L S L P E *

TGTTCCTGCATCTCCCCTCAGATTCCACCTAGAGCTAGAATGTGTCTTACCAGATCTAATA 1620

TTGACTGCCTCTGCCTGTCGCATGAGAACATTAACAAAGCAATTGTATTACTTCCTCTG 1680

TTCCCGACTAGTTGGCTCTCAGATACTAATAGGTGTGTCAGGCTCCGGATCTTTCTGGAA 1740

TTCATATTGAATGATGTGATACAAATTCATAGTCAATATCAAGCAGTGAAATATGATAAT 1800

AAAGGCATTTCAAAGTCTCACTTTTATTGATGAAAATAAAAATCATTCTACTGAACAGTCC 1860

ATCTTCTTTATACAATGAGCCACATCCTGAAAAGGGTGTTGCTAAGCTGTAACCGATATGC 1920

ACTTGAAAATGATGGTAAGTTAATTTTGATTCAGAATGTGTTATTTCTCACAAATAAACAT 1980

AATAAAAGGGAAAAAAAAAAAAAA 2003

FIGURE 45 (3 of 3)

(SEQ ID NO: 167)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07491

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :435/6; 530/350; 536/22.1; 424/88

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 530/350; 536/22.1; 424/88

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
NONE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 September 1993	1 5 OCT 1993

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	DAVID R. PRESTON
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-20
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

   Claims 1-20 refer to sequence ID information which is so extensive that without a computer readable format a reasonable search may not be performed.

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
   (Telephone Practice)
   I.   Claims 1-20 drawn to a protein and first process of using, inhibition of cellular proliferation.
   II. Claims 21-22, drawn to a second process of using, a method of making an antibody.
   III. Claims 23-24, drawn to a third process of using, a specific binding assay.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
   1-20

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

   ☐ No protest accompanied the payment of additional search fees.

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C12Q 1/68; C07K 3/00, 13/00, 15/00, 17/00; C07H 19/00, 21/00; A61K 39/00